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VITAMINS AND HORMONES

VOLUME VII

VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

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VOLUME VII

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EDITORS' PREFACE

The reward for the tedious work of an editor of a scientific book comes from the expressions of appreciation by his colleagues. On this account the task of the Editors of *Vitamins and Hormones* continues to be particularly satisfying.

In this seventh volume are included seven articles on the subject of vitamins and five on hormones. The articles on anterior pituitary hormones are especially significant because they contain considerable data heretofore unpublished, representing a marked advance in our knowledge of protein hormones. Similarly, the extensive infrared spectrographic data on the steroids comprises partially unpublished reference material of great value.

With this volume we complete the group of articles on the nutritional requirements of the more important laboratory animals: primates other than man (Volume II); chick and mouse (Volume V); cotton rat and hamster (Volume VI); rat and guinea pig (Volume VII).

It is to be hoped that the present volume will be found as useful as its predecessors. Criticisms and constructive suggestions for future policy will be warmly welcomed.

ROBERT S. HARRIS
KENNETH V. THIMANN

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Vitamin P

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I. CAPILLARY RESISTANCE AND NUTRITIONAL STATUS

1. Introductory

Since the studies of Hess and Fish in 1914, it has been held that capillary strength is associated specifically with the intake of ascorbic acid. It is inherent in this view that scurvy, and deficiency states of ascorbic acid short of clear-cut scurvy, are marked by unduly fragile capillaries. This is still the general view today, in spite of a considerable body of evidence to the contrary which, taken together, seems overwhelming.* The subject has been reviewed in more detail recently by Munro *et al.* (1947), and it is, therefore, unnecessary to reopen the matter again. We agree with their conclusion that "there is very little evidence to associate capillary strength specifically with vitamin C intake in man" and would only add that this statement applies also the guinea pig.

In 1936 Armentano *et al.* reported that Hungarian red pepper and lemon juice contained a factor that could increase capillary strength and reduce capillary permeability in man, but was not ascorbic acid. As a result of animal experiments it was believed that the material responsible for these effects was a vitamin, and, because of its apparent effect on permeability and its presence in paprika, it was provisionally named vitamin P (Permeabilitäts-Vitamin). This claim has since been questioned on two counts, the one that no such substance exists, and the other that its action is pharmacological rather than that of a vitamin.

It is our purpose in this review to consider the evidence bearing upon these points and to summarize what is known about the chemical constitution and physiological properties of many of the materials which have now been reported to produce similar biological effects. The number and variety of these materials compel the view that the term vitamin P embraces a group of compounds not all of which are closely related chemically.

2. Capillary Permeability and Capillary Resistance

The term *capillary permeability* has frequently been used loosely in medical literature. Strictly speaking it should be restricted to that property of the capillary endothelium which is measured in terms of the volume of material passing per unit area per unit time per unit thickness per unit of effective pressure. For clinical purposes, however, such a definition is too rigorous, and capillary permeability may be thought of in terms of the volume of fluid filtered per unit mass of tissue per unit time. Of two methods available for assessing capillary permeability in man, that of Landis *et al.* (1932) was used by Armentano and his colleagues in spite of the fact that its limitations for this purpose had been clearly demonstrated by Landis *et al.* Although it is possible to demonstrate filtration of fluid in the human forearm when the outflow of venous blood has been restricted, the results of successive observations in the same individual are such as to make real differences most difficult to evaluate (see for example Bing, 1938). So far as we know, the second method (Landis and Gibbon, 1933) has not been applied to studying the effect of vitamin P preparations on capillary permeability.

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* For example: Abt *et al.* (1936); Bell *et al.* (1940); Brante (1939); Crandon *et al.* (1940); Difs (1940); Fox *et al.* (1940); Greene (1934); Goettsch (1935); Göthlin (1937); Heinemann (1941); Holland *et al.* (1947); Levcowich and Batchelder (1942); Liebmann *et al.* (1938); McNee and Reid (1942); Molitch (1935); Munro *et al.* (1942); O'Hara and Hauck (1936); Rapaport *et al.* (1940); Scarborough (1940, 1943); Scarborough and Gilchrist (1944); Turpeinen (1943); Weld (1936).

It is furthermore important to distinguish between capillary permeability and *capillary fragility,* which involves the passage of erythrocytes outside the vessels, either through the intercellular cement substance or as a result of the actual rupture of capillary walls. In the literature these terms have frequently been used synonymously whereas in fact the dissociation between them is often striking. Thus, in the various forms of purpura there is ample evidence of the presence of erythrocytes outside the vascular system (purpura), but no sign of the accumulation of fluid (edema). On the other hand tissue damage produced by heat or cold may result in considerable swelling of the injured area and the production of excessive tissue fluid, containing a high concentration of protein without any purpura. Until, therefore, more direct observations of both capillary permeability and capillary fragility have been made simultaneously in the same area, it is probably wise to consider permeability and fragility as different properties of the capillary wall and to judge vitamin P activity on the basis of effectiveness in increasing the capillary strength. For these reasons, and because of the doubt which surrounds many so-called determinations of capillary permeability in the clinic, we shall not refer to such studies again. That gross disturbances of capillary permeability and capillary fragility may occur simultaneously is, however, witnessed by cases of hemorrhagic whealing, where not only fluid rich in protein but also erythrocytes in large numbers are found in the hemorrhagic vesicles.

3. *Tests of Capillary Resistance*

The terms *capillary resistance,* capillary fragility, and capillary strength may be used interchangeably. The first, suggested originally by Hess, is the more generally used by workers in this field and is, on the whole, to be preferred because this property of the capillary walls is most conveniently examined by determining their ability to resist pressures applied to them. Apart from certain procedures of limited usefulness such as the snake venom reaction of Peck *et al.* (1936) and the "flicking test" of Jones and Tocantins (1933), all tests for assessing capillary resistance may be classified as either positive or negative pressure tests. Positive pressure tests are based upon the fact that when the venous return from a part (limb) is obstructed the pressure within the capillaries below the obstruction rises rapidly. In negative pressure tests the capillaries are suddenly submitted to sub-atmospheric pressures applied to them through the skin. In both types of test the stress applied is sufficient to rupture capillaries, and the effect becomes evident as minute hemorrhages (petechiae) in the skin. The ease with which such petechiae can be produced serves as a measure of the strength of the capillaries (capillary resistance). It is clear that such tests are confined to the skin by the nature of the procedure.

It is not desirable here to consider in detail the merits and demerits of the many different methods that have been advocated for assessing capillary resistance, especially as the more commonly used have already been briefly dealt with by Munro *et al.* (1947). In the literature the two types of tests are referred to indiscriminately as though they measured the same thing. There is, however, evidence that this is not entirely true. Thus, Bell *et al.* (1942), using the Göthlin positive pressure test which involves venous obstruction for 15 minutes (Bell *et al.* 1940) and the negative pressure method described by Scarborough (1941b), found the correlation between results of the two tests to be barely significant for 142 healthy medical students. Scarborough (unpublished) has also examined the correlation between a rapid positive pressure test (venous obstruction for 5 minutes) and his negative pressure test in 120 healthy adolescents and has obtained correlation coefficients of under −0.3. This is in line with the experience of other workers (see Munro *et al.* 1947) and indicates that care must be exercised in comparing the results obtained by one method with those provided by another. It also increases one's confidence in a conclusion based upon results obtained by both types of test. It is necessary to refer to these discrepancies here because they may account, in part at least, for the different impressions of different workers as to the efficacy of preparations of vitamin P in elevating capillary resistance.

Even when these technical difficulties have been given due regard, there are still several factors for which allowance must be made in evaluating the results of both types of test. Although some of these were appreciated by early workers in this field (for example, Öhnell, 1928), they have frequently not received adequate attention. Among the normal physiological factors that may have to be considered, particularly with negative pressure methods, are diurnal and seasonal differences and variations due to altitude, humidity, age, exercise, skin temperature and menstruation, as well as alterations determined by differences in texture of the skin, method of application of pressure and part of body in which the test is made. Inflammation, fever, hypertension, certain metabolic disorders, such as hyperthyroidism and diabetes and conditions associated with cutaneous erythema may all influence the results of capillary resistance tests. It is possible that differences in capillary resistance produced as the result of one or more such factors may be responsible for some of the so-called spontaneous variations by which certain workers have been surprised and discouraged. To add to the difficulties of those who approach the problem of vitamin P assay and the study of nutritional or hematological disorders associated with bleeding, there are two other factors which must be taken into account. The first is that any increase in capillary resistance produced as the result of the administration of an active substance is related to the initial level of capillary resistance before dosing (Scarborough and Wokes, 1946). The second is that the extravasation of blood into the tissues is itself followed by a sharp elevation in capillary resistance, which may be sustained for 3 or 4 days (Scarborough, 1941a).

4. Capillary Resistance and Scurvy

Before referring to the evidence for the existence of vitamin P, it is well to consider the background against which the results of more detailed studies should be viewed. The majority of workers have observed that in poorly nourished subjects the capillary resistance is significantly lower than in persons living on adequate diets, whether the tests be made by the positive or the negative pressure method. Although it is true, as emphasized by Munro *et al.* (1947), that capillary resistance is "normal" in at least two-thirds of undernourished subjects, this observation serves at least as well to suggest the desirability of re-evaluating what is to be correctly regarded as a normal capillary resistance as to discredit the facts referred to above, demonstrated so clearly by numerous observers at different times, using different techniques, in groups of healthy school children, children in institutions, students, healthy adults, hospital patients and old men. In frank scurvy positive pressure tests indicate unduly fragile capillaries in some 60 to 70% of cases, and Scarborough (unpublished) has found low capillary resistance in approximately 60% of 73 cases of scurvy by his negative pressure method. An observation that may, in part, account for the fact that a low capillary resistance is not invariably found in scurvy has been made by Scarborough (1941a), who reported that the extravasation of blood into the tissues can elevate capillary resistance.

That the low capillary resistance found in a significant proportion of subjects living on diets containing inadequate amounts of fruit and vegetables cannot be related to their impaired intake of ascorbic acid is strongly suggested by the following facts:

(a) There is no correlation between the plasma concentration of ascorbic acid and capillary resistance as determined by either positive or negative tests.

(b) There is no significant relation between capillary resistance, measured either by positive or by negative pressure methods, and the results of saturation tests with ascorbic acid.

(c) We have never been able to detect any increase in capillary resistance following the administration of ascorbic acid alone to apparently healthy persons living on inadequate diets or to cases of frank scurvy. Most reports from other workers confirm our experience.

The effect of modifying the diet, as, e.g., by administration of fruit and fruit juices, in elevating the low capillary resistance found in persons subsisting on inadequate amounts of fruits and vegetables is not easy to gauge because the majority of such studies are not adequately controlled. It seems likely, however, that in some 60% of them the capillary resistance has been elevated by such means.

A reasonable interpretation of the results of all these studies is that a diet rich in fruit and vegetables contains some substance or substances that can maintain capillary resistance, and that in its absence capillary resistance tends to be low. On the basis of present concepts of the 'normal' some 60 to 70% of persons living on diets inadequate in this material exhibit signs of deficiency as revealed by unduly low capillary resistance. This substance is not ascorbic acid but, since it occurs in many foods which are themselves sources of ascorbic acid, evidence of ascorbic acid deficiency may often be expected in association with a low capillary resistance. The fact that frank scurvy may be present in certain individuals along with a capillary resistance that is not abnormally low may then be regarded as an expression of the balance between their needs and their intakes of the two materials. Whether this explanation or that advanced above be the more important in accounting for a high capillary resistance in certain cases of scurvy cannot be decided until the behavior of the capillary resistance during the onset of scurvy has been more closely studied. It may be mentioned, however, that Crandon, *et al.* (1940) found no change in capillary resistance by either a positive or a negative pressure method during the development of experimental scurvy in one subject and yet it is difficult to point to any source of vitamin P in their experimental diet.

5. "Citrin" and Hesperidin in Therapy

In 1936 Armentano (a and b) described in patients with bleeding diseases favorable therapeutic effects from the use of lemon juice and preparations of paprika ("vitapric") rich in ascorbic acid. Armentano *et al.* (1936) were struck by the observation, however, that similar results could not be obtained with pure ascorbic acid. Considering, therefore, that some other active material must have been present in the fruit, they were able to obtain by repeated lead and barium precipitation an active fraction which was finally crystallized from methanol in a yield of about 2 g. from 200 kg. of lemons, or 70 liters of lemon juice. This material, which was believed to contain a mixture of flavones, was called "citrin" (citrus flavone). It was used in the treatment of seventeen patients, many of whom had a low capillary resistance as measured by a negative pressure test (Borbély, 1930). In four cases of thrombocytopenic purpura and in two of diabetes, "citrin" was without effect on capillary resistance, but in three cases of vascular purpura (with normal thrombocyte counts), one of myxedema and seven of various forms of infection, the capillary resistance was increased in all but one. In the cases of vascular purpura there was, in addition, a pronounced amelioration of the disease. In many of the more recent clinical studies on the effect of "citrin" and similar materials on capillary resistance and the bleeding diseases, due allowance has not been made for spontaneous variations in capillary resistance and spontaneous remissions of the disease. It is therefore well to emphasize that Armentano *et al.* were alive to this possibility, and by their selection of chronic cases and repeated determinations of capillary resistance anticipated this criticism of their work. There is no information in their report to show whether the patients were on inadequate diets before and during the treatment, but it is clear that in several cases the capillary resistance, although elevated by the administration of "citrin," was not abnormally low before treatment began. Bell *et al.* (1940) were unable to show that vitamin P (hesperidin) significantly increased the capillary resistance of apparently healthy persons with low capillary resistance (positive pressure test).

Struck by the apparent effect of hesperidin (a flavanone glycoside possessing vitamin P activity) in arresting the subcutaneous bleeding in three cases of arsenic or bismuth intoxication occurring in patients who had subsisted on inadequate diets for prolonged periods, Scarborough and Stewart (1938) studied the effects on capillary resistance (positive pressure method) of repeated periods of hesperidin administration in three cases with multiple vitamin deficiency disorders. The initial capillary resistance in all three was low before treatment began and was elevated in each by hesperidin. Discontinuance of hesperidin treatment was followed by a fall in capillary resistance. Similar results (Scarborough, 1940) were obtained in five cases, all with evidence of vitamin deficiency disorders and all with low capillary resistance (positive pressure test), again (Scarborough, 1945) in nine similar cases (negative pressure test) and in two cases of scurvy (1944, negative pressure test). The only other workers who appear to have studied the effect of vitamin P preparations in cases of nutritional deficiency are Lazarus *et al.* (1948), using the negative pressure method of Scarborough: they got slight and inconsistent increases in capillary resistance following the administration either of ascorbic acid or of preparations of vitamin P.

The observation of Armentano and his colleagues that vitamin P preparations are capable of increasing capillary resistance in cases of vascular purpura has been repeated by Rusznyák (1937), Armentano (1940), Barcaglia (1940), Scarborough (1942a and unpublished cases) and Parrot *et al.* (1944). In allergic purpura, elevation of capillary resistance following treatment with vitamin P has been described by Jersild (1938), Hiramatsu (1939) and Kugelmass (1940). In other patients with unduly fragile capillaries, vitamin P preparations have also elevated capillary resistance (Jersild, 1939; Horne and Scarborough, 1940; Rapaport and Klein, 1941; Vacek, 1941; Griffith *et al.*, 1944; Shanno, 1946). Finally Hiramatsu (1941a) has reported greatly diminished bleeding after intraperitoneal hesperidin administration (50 mg. doses) in both thrombopenic and vascular purpura, especially the latter, produced by injection of antiplatelet and antiendothelial sera, respectively, into guinea pigs. These studies suggest that preparations of vitamin P can elevate the low capillary resistance found in persons subsisting on inadequate diets and in patients suffering from certain forms of purpura. That the action of such preparations is that of a vitamin has not been shown so clearly.

6. *Effect of Vitamin P on Scorbutic Animals*

The original suggestion that the action of "citrin" was that of a vitamin was based on observations of Armantano *et al.* (1936) that a group of scorbutic guinea pigs given the flavone preparation outlived a control group not given "citrin." Bentsáth *et al.* (1936, 1937) later reported that the survival time of guinea pigs fed the Sherman-La Mer-Campbell scorbutogenic diet was increased from an average of 28.5 to an average of 44 days when supplements of vitamin P ("citrin") were added to the diet. At autopsy the animals which had received "citrin" showed significantly fewer tissue hemorrhages than did their controls. It was concluded, therefore, that scurvy as ordinarily produced in the guinea pig represents a combined deficiency of ascorbic acid and vitamin P. Zacho (1939) and Todhunter *et al.* (1940) also reported that the amount of hemorrhage at autopsy was less in guinea pigs receiving sources of vitamin P than in animals maintained on the same scorbutogenic diet without supplements. It has, however, been shown by Zilva (1937) that the post-mortem findings described by Bentsáth *et al.* in the animals that developed scurvy on a scorbutogenic diet plus supplements of vitamin P closely resemble the condition found in guinea pigs receiving minute doses (0.1 mg. daily) of ascorbic acid as supplements to a scorbutogenic diet. In dietary experiments with guinea pigs neither Zilva (1937) nor Moll (1937) could find any evidence for the existence of a vitamin P and the Hungarian group (Bentsáth and Das, 1937) and Szent-Györgyi (1937, 1938) have subsequently reported failure to repeat the guinea pig experiments upon which the discovery of a capillary resistance-increasing vitamin was principally based. Inability to delay or modify the onset of scurvy in guinea pigs by giving them supplements of vitamin P has been noted also by Detrick *et al.* (1940), Parrot *et al.* (1946a) and Hiramatsu (1940). The idea that vitamin P can modify the course of scurvy in the guinea pig, and that this disorder is due to a mixed deficiency of ascorbic acid and vitamin P, must therefore be discarded. It receives no support from clinical observations (for example, Scarborough, 1940).

In none of the above studies were any measurements made of capillary resistance. Zacho (1939) observed that in guinea pigs maintained on a scorbutogenic diet the capillary resistance fell progressively. The initial normal levels could not be restored until supplements of vitamin P ("citrin") were added to the diet. Bacharach *et al.* (1942) also found a fall in capillary resistance in guinea pigs maintained on scorbutogenic diets supplemented with ascorbic acid. This lowered capillary resistance was elevated by vitamin P, which was found in many fruits and vegetables (Bacharach and Coates, 1943) after these studies had led to an acceptable method for the biological assay of vitamin P potency (Bacharach and Coates, 1942). Using a slightly different technique, Bourne (1943b) has also shown that there was a marked fall (of at least 50%) in the capillary resistance of 54 guinea pigs on a scorbutogenic diet supplemented with 10 mg. per head per day of ascorbic acid. After five daily doses each of 10 mg. of "citrin" all fifteen animals tested showed increases in capillary resistance of between 60 and 100%. In those animals to which no "citrin" was given the capillary resistance remained at its low level. When citrin treatment was discontinued the animals which survived developed low capillary resistance again in 1 to 4 weeks. This work represents the most convincing evidence for the existence of a substance or group of substances which increases capillary resistance and strongly suggests that the action is that of a vitamin, at any rate for the guinea pig. Bourne's observations have been confirmed by Hughes and Parkes (1946) who found that in guinea pigs on a diet lacking greenstuff, but to which was added 5 mg. of ascorbic acid per animal per day, initially there might be no response to a negative pressure of 600 mm. Hg, though, minimum values of 200–350 mm. Hg were obtained in from 3 weeks to 4 months. The initial figures were restored by many different flavanones. Todhunter *et al.* (1940) also observed that on a scorbutogenic diet to which supplements of ascorbic acid were added the capillary resistance fell considerably and that normal levels were restored by the administration of vitamin P. Parrot *et al.* (1946b) studied ten guinea pigs given a diet containing bran, oats, bread and water, supplemented with B vitamins, cod liver oil and 50 mg. ascorbic acid daily per animal. On this diet a fall in capillary resistance was observed within 27 days. The daily supplement of ascorbic acid was then reduced to 0.4 mg. and five of the animals were given vitamin P (1 mg. of *d*-epicatechin per head three times weekly). All the animals developed scurvy, but the low capillary resistance persisted only in those receiving no vitamin P. This work is of particular interest in that scurvy was produced in animals with a high capillary resistance. Rusznyák and Benkó (1941a and b) have also confirmed the observations of Zacho that the capillary resistance of guinea pigs on a scorbutogenic diet is restored to normal only by preparations of vitamin P. By giving a scorbutogenic diet to rats they obtained a considerable lowering of capillary resistance in 5 to 6 weeks, which was elevated to normal in 10–14 days on the subcutaneous administration of 3–4 mg. "citrin" per day. Majovski *et al.* (1944) devised a procedure whereby mice were submitted suddenly for 1 minute to a low atmospheric pressure (70 mm. Hg). Very few animals survived this procedure, but the authors reported that the amount of hemorrhage in the lungs was significantly less in groups of mice receiving vitamin P (crude hesperidin, eriodictyn, hesperidin chalcone). These results could not be repeated, however, by Kibrick and Goldfarb (1944), who regarded the method as unsatisfactory for assessing the potency of preparations of vitamin P. In a brief report, Thienes *et al.* (1942) state that the capillary resistance of rabbits is diminished when the animals are maintained on a scorbutogenic diet, and that normal values are restored by the administration of vitamin P.

Lavollay and Sevestre (1945) and Wilson *et al.* (1947), both using guinea pigs and negative pressure methods for determining capillary resistance, have not been able to repeat Zacho's results; the former group could find no fall in capillary resistance when the animals were fed a scorbutogenic diet supplemented with ascorbic acid and the latter, in similar experiments, could find no relation between capillary resistance and the oral administration of vitamin P (rutin). The discrepancies between experience in different laboratories, and in different clinics, with capillary resistance tests is confusing and unfortunate, because a simple method of assaying vitamin P potency is urgently required. We still hold, however, that solution of these discrepancies and further studies of capillary resistance offer one of the most hopeful lines of investigation.

To set against the work referred to above, two further reports must be mentioned. Matusis (1937) observed a decrease in capillary resistance of guinea pigs receiving a scorbutogenic diet. This was unaffected by the administration of "citrin" prepared by the method of Szent-Györgyi (1938). McHenry and Perry (1940) also state that preparations of vitamin P are without effect in preventing the fall in capillary resistance which occurs in scorbutic guinea pigs. Both groups of workers observed an elevation of capillary resistance following treatment with ascorbic acid, and they therefore conclude that the changes in capillary resistance are correlated with ascorbic acid intake.

In spite of these two reports, which are difficult to assess because of the lack of detailed information about the experiments, the present position may be summarized by saying "it is, however, generally agreed that guinea pigs placed on a scorbutic diet supplemented with adequate amounts of ascorbic acid show a decline in capillary strength, as measured by negative pressure methods, and that capillary strength is restored by administration of vitamin P." This conclusion was arrived at by Munro *et al.* (1947) in reviewing the value of tests of capillary strength for the diagnosis of ascorbic acid and vitamin P deficiencies in man. It is in line with the evidence presented above.

These observations lead one to consider the question of a deficiency syndrome in animals and man. Armentano *et al.* (1936) were the first to point out that a study of guinea pigs on a scorbutogenic diet would be unlikely to result in any great differences between animals receiving sources of vitamin P and a group not doing so, because in such experiments the effects of total lack of ascorbic acid rapidly dominate the picture. They also point out, and it is still true, that there is no certain method of preparing a diet free from all sources of vitamin P. More recently Parrot *et al.* (1946a) have stated that certain diets allegedly free from vitamin P in fact contain sources of the vitamin and Zacho (1939) stated that he found it impossible to devise a diet completely free from flavanones. His scorbutogenic diet proved on chemical examination to contain small amounts of these compounds. Moreover, it is stated by Robeznieks (1938) that hesperidin, reported by many workers to possess vitamin P activity, after being autoclaved in 10% ammonium hydroxide for 1 hour at 120°C. still gives a cyanidin reaction (*vide infra*) of the same intensity as before heating. This observation may be of some importance since it has been assumed that flavanones and flavones in the diet can be destroyed by autoclaving in ammonia fumes. It is important to recall in this connection that Hughes and Parkes (1946) state that their guinea pigs receiving a scorbutogenic diet with 5 mg. per head per day of ascorbic acid by mouth, did not show minimal values of capillary resistance until after from 3 weeks to as long as 4 months. Even at this time some animals showed little fall. It seems reasonable to assume, therefore, that any deficiency syndrome that it might be possible to produce in guinea pigs maintained on such a regimen would take a longer time than this to develop. Similarly, in Bourne's experiments, the shortest time for the capillary resistance to fall was 3 weeks and the longest 8 weeks. Although Rusznyák and Benkó (1941) stated that rats fed a scorbutogenic diet showed a diminished capillary resistance, which is raised by vitamin P, no hemorrhagic disorder has yet been reported in this animal, in spite of the numerous occasions when rats have been maintained for long periods on synthetic diets. Zacho (1939) found that guinea pigs on scorbutogenic diets supplemented with ascorbic acid exhibited a gradual fall in capillary resistance, but continued to gain in weight and showed no ill health, and Slanetz (1947) reported that guinea pigs on a scorbutogenic diet supplemented with ascorbic acid can breed successfully. Munro *et al.* (1947) point out that there is no satisfactory evidence that lack of vitamin P is harmful to guinea pigs in spite of the lowered capillary resistance. It must be remembered, however, that guinea pigs on a diet completely devoid of ascorbic acid continue to gain weight and apparently thrive until frank scurvy develops and that this occurs quite suddenly (see, for example, Zilva, 1937). It may be, therefore, that experiments in which guinea pigs have been maintained on scorbutogenic diets supplemented with ascorbic acid have not been continued long enough to allow a clinical syndrome due to deficiency of vitamin P to develop or that the diets used contained unsuspected vitamin P. Apart from Parrot *et al.* (1946a), who state that the development of articular swellings and thyroid and adrenal hypertrophy in guinea pigs receiving only traces of ascorbic acid is prevented by supplements of *d*-epicatechin, no one seems yet to have produced a vitamin P deficiency syndrome in any species of animal. It must be admitted, however, that a properly conducted experiment in which guinea pigs are given a scorbutogenic diet supplemented with adequate amounts of ascorbic acid and observed over a period of at least 6 months with repeated determinations of capillary resistance has not yet been reported.

7. *Spontaneous Vitamin P Deficiency in Man*

That clinical signs of a vitamin P deficiency can occur in man has been claimed by Scarborough (1940), who reported the experimental production of a syndrome characterized by pains in the legs on exertion, pain across the shoulders, weakness, lassitude and fatigue associated with greatly impaired capillary resistance and the development of spontaneous hemorrhages in areas exposed to pressure. These hemorrhages were all small (petechiae) and they involved particularly the hair follicles. No abnormality of the blood was detected. The symptoms were abolished, the hemorrhage arrested and the capillary resistance elevated to normal by the administration of preparations containing vitamin P. This syndrome was produced in two elderly male patients admitted to the hospital with frank vitamin deficiency disorder, and maintained thereafter for 4 months on a vitamin deficient diet, supplemented with vitamin A, vitamin D, thiamine and ascorbic acid in large doses. From these observations and a study of the value of vitamin P and ascorbic acid in the treatment of scurvy (Scarborough, 1940; 1944), it is argued that at least two forms of subcutaneous bleeding may occur in man as the result of defective diets. One, scurvy, due to deficiency of ascorbic acid, is promptly controlled by the administration of ascorbic acid but not of vitamin P. It is not invariably accompanied by a low capillary resistance. However, since present knowledge indicates that ascorbic acid and vitamin P frequently occur together in natural sources, it is not surprising that scurvy is frequently found associated with a low capillary resistance. In an individual living on an inadequate diet a low capillary resistance is regarded as an expression of a deficiency of vitamin P and is elevated promptly by administration of this vitamin but not by ascorbic acid. Spontaneous petechial bleeding and other symptoms may develop when the capillary resistance reaches very low values. They are also evidence of vitamin P deficiency and are speedily controlled by administration of the vitamin. The two forms of hemorrhage are different in appearance. The hemorrhages characteristic of scurvy are large (ecchymoses), often involving considerable areas of subcutaneous tissue and muscle together with bleeding from the gums. The hemorrhages developing as the result of vitamin P deficiency are small (petechiae) and, so far as clinical examination can tell, occur in the skin, often being confined to hair follicles. Both forms of hemorrhage tend to appear first, and are usually more extensive, in parts exposed to the pressure of clothing and in the legs, because of the higher venous pressure there. Although petechial bleeding in association with a strikingly low capillary resistance has been observed in a third case (of scurvy) after treatment with ascorbic acid as supplement to a vitamin-deficient diet, these studies have not been repeated by any other worker. They are criticized by Munro *et al.* (1947) on the grounds that the two published cases received a preparation of vitamin P, which temporarily elevated capillary resistance, during the induction of the syndrome. Scurvy, however, may be produced in guinea pigs receiving small amounts of ascorbic acid every day (Zilva, 1937, 0.1 mg. and 0.2 mg. daily; Todhunter *et al.* 1940, 0.47 mg. and 1.8 mg. daily). A deficiency syndrome is the end result of a gross discrepancy between supply and demand rather than the expression of a complete lack of the specific vitamin. Levcowich and Batchelder (1942) made repeated observations by a negative pressure method of the capillary resistance of two women. After the nineteenth week on a basal diet which apparently contained little ascorbic acid, but which was supplemented with ascorbic acid at various levels, they observed that the petechiae induced at each testing, while not more numerous, became progressively larger. Shortly after a return to a diet containing plentiful fruit and vegetables, the induced hemorrhages again became small. Levcowitch and Batchelder tentatively suggested that the larger hemorrhages might be an early sign of vitamin P deficiency.

It is of interest here to recall the patient described by Jersild (1939); she had increased capillary fragility and subcutaneous bleeding of the ecchymotic type. Intensive therapy with ascorbic acid was ineffective and she was subsequently cured with intravenous "citrin," 50 mg. daily for about a month, which elevated capillary resistance. Two cases described by Elmby and Warburg (1937) with prolonged intestinal disorders had intractable bleeding from the bowel and this ceased only when lemon juice was given, synthetic ascorbic acid having proved valueless. Simultaneously the capillary resistance, which had been much reduced, was conspicuously elevated. In all these patients the diet had been inadequate for many years.

Critical appraisal of the situation, then, justifies the conclusion that there exist materials capable of elevating capillary resistance both in laboratory animals and in man. There is satisfactory evidence, derived from both the guinea pig and from man, that such substances are normal dietary constituents and that a fall in capillary resistance is associated with deficiency. It still has to be established, however, that abnormally fragile capillaries confer any disadvantage on the organism. An experimentally produced clinical syndrome due to deficiency of vitamin P has been described in man but no confirmation of these studies has yet been reported. No one has described such a deficiency in an animal, but a completely satisfactory attempt to produce it has evidently not been made. The fact that many workers have reported that preparations of vitamin P produce increases in the abnormally low capillary resistance of patients with a variety of disorders, but in whom unequivocal evidence of dietary deficiency was not obtained, cannot be cited as evidence against the vitamin character of the preparations. To explain these findings it is not unreasonable to postulate factors, for example infection, which increase the daily requirements of the vitamin. Until some reasonably reliable estimate can be made of the daily intake necessary to maintain capillary resistance at a certain level one cannot begin to study such factors.

II. VITAMIN P ACTIVITY AND CHEMICAL CONSTITUTION

1. Phenyl Benzo-γ-Compounds (Flavonoids)

Before entering into any detailed consideration of the "chemistry of vitamin P," that is to say, the structure of those compounds that have been reported to show vitamin P activity, it is necessary to stress one point. There are scattered throughout the literature of the last 10 years statements that this, that or the other plant material, product, concentrated extract or chemical compound (whether of natural or laboratory origin) shows vitamin P activity. Some of these claims will not stand critical examination. The fundamental test—the one that establishes in a substance the ability to reverse capillary fragility due to a dietary deficiency—must be carried out on a species of animal that is known to be susceptible to that deficiency. If some chemical reaction or physical property has been found to show a high degree of correlation with this biological activity, it may be used as a sorting test, without prejudice to the possibility, however slight, that the response of vitamin P deficient animals or men to it may nevertheless not confirm the indirect test. On the other hand, when a chemical reaction that merely happens to characterize one particular compound or group of compounds having vitamin P activity is undiscriminatingly used to determine the "vitamin P content" of a range of materials, the results are valueless, unless it has previously been shown that many substances with vitamin P activity all give the reaction and that no substances devoid of the activity will. If, therefore, in what follows nothing is said of certain compounds claimed to increase capillary resistance, it will be because the claim was accompanied by no valid evidence to show that it had ever done so, or indeed that anyone had even tried to make it do so.

Although the various substances with which we are here concerned—those, that is to say, that may be held to have been legitimately characterized as single substances—do not fall within any simply named group of compounds, they have certain features in common. All have a skeleton consisting essentially of a benzenoid ring fused to a γ-pyrone ring; this may be wholly or partly reduced and a further benzenoid ring is attached to the carbon atom adjacent to the ring oxygen.

An important characteristic of some of these compounds is the tendency of the ring to be split between the oxygen atom and the carbon atom at position 2. The resulting chalcone (chalkone) is a strong reducing agent and also tends to pass back into the cyclic and (generally) much less water-soluble form. One of the several theories put forward about vitamin P activity has been that it is a property only of the chalcones and not of the cyclized compounds.

Formula I thus represents the skeleton of most, if not all, of the naturally occurring substances that have been found to show vitamin P activity. All of them contain four hydroxyl or substituted hydroxyl groups, in the 5, 7, 3′ and 4′ positions. When the 2:3 double bond is reduced the compound is a flavanone. Eriodictyol (formula II) is thus 5, 7, 3′, 4′-tetrahydroxyflavanone: if the hydroxyl group at 4′ is methylated, the substance is hesperetin, the aglycone of hesperidin, which carries a gluco-rhamnose residue at position 7 (formulas II and III). The finding of Javillier and Lavollay (1946) that phloretol (formula VII) is strongly active makes untenable the view, suggested by Szent-Györgyi himself, that vitamin P activity is associated with the two adjacent phenolic groups in the 3′ and 4′ positions of the phenylbenzpyrone molecule, for only the 4′ group is present in phloretol.

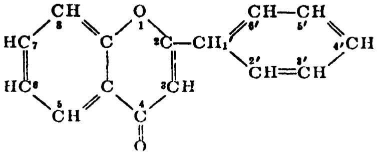
Another variation on the phenylated chromone theme results from the presence of a hydroxyl group at carbon atom 3. This gives a flavonol, which may exist in tautomeric forms (formula IV). If then the 5, 7, 3′ and 4′ carbon atoms are again furnished with hydroxyl groups, the result is the tetrahydroxy flavonol, quercetin, the aglycone of at least two naturally occurring glycosides. One of these, rutin, has glucose and rhamnose as a disaccharide residue attached at the 3-hydroxy group (formula V): in this compound, it will be observed, the possibility of isomerism like that shown in formula IV no longer exists. If there is reduction of the 2:3 double bond and also hydroxylation in the reduced ring, the resultant compound, which now has an asymmetric carbon atom at position 3, is properly designated a flavanol, though the term cannot indicate whether the hydroxyl group is at position 4 or at 3, as in the catechins (formula VI). It should be noted, moreover, that the catechins are not γ-pyrone derivatives, owing to reduction of the ketonic oxygen at position 4: they are rather to be regarded as representing a condensation of the benzene and dihydropyran rings.

2. Active Substances

It is, in our view, unfortunate that many authors have been far from scrupulous in assigning their correct chemical description to some of these compounds and have so confounded further an already sufficiently confused story. Thus Griffith *et al.* (1944)—to take an example at hazard, and by no means because these authors are solely or exceptionally guilty—write "Rutin is, like hesperidin, a flavone glucoside." In fact, neither

A. FLAVONE GROUP

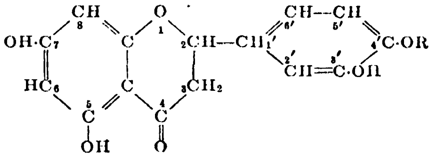
I.



2-Phenyl-1:4-benzopyrone
(phenylchromone)

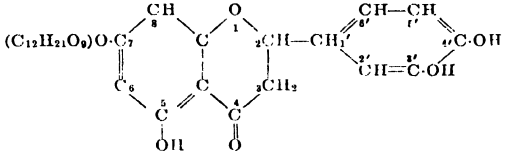
B. FLAVANONE GROUP

II.



R = H Eriodictyol
R = CH_3 Hesperetin

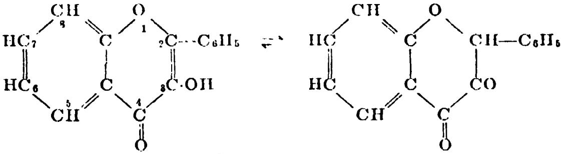
III



Hesperidin

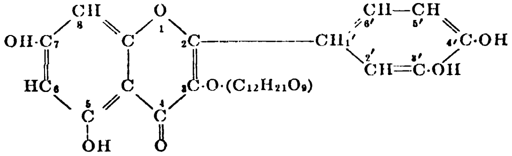
C. FLAVONOL GROUP

IV



2-Phenyl-3-hydroxy-1:4-benzopyrone

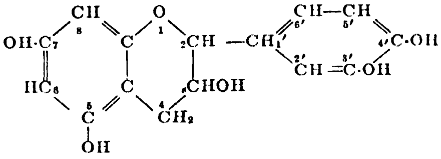
V.



Rutin

D. CATECHIN GROUP

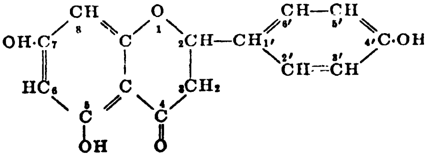
VI.



Catechin and
Epicatechin

E. PHLORETOL

VII.



is a glucoside, for the sugar moieties are not glucose, but rhamnoglucose, and neither is a flavone derivative; rutin is a flavonol glycoside and hesperidin a flavanone glycoside, as we have seen above.

We can turn now from this consideration of structural formulas to note some of the compounds for which experimental evidence can be adduced to justify their consideration, in the light of what we have written above, as having vitamin P activity. Although some authors have referred (generally in passing only) to the proven inactivity of hesperidin, we are in no doubt that this is as little to be accepted as the many statements attributing vitamin P activity to compounds of the flavone type on the grounds that they give a color reaction with boric acid (C. W. Wilson, 1939). We are satisfied that, under suitable conditions, guinea pigs can be made to show a reduced capillary resistance not raised by ascorbic acid but raised by hesperidin, recrystallized to constant specific rotation and optical absorption, and given orally. We have also found the same material to be active in the treatment of that condition, discussed earlier in this review, sometimes to be found in cases of human scurvy "cured" with pure ascorbic acid. It is true that, as we point out below, there are other substances showing much greater activity than hesperidin, or than purified rutin, which in our hands has given results of the same order as hesperidin, both on guinea pigs and on man. The very low solubility of hesperidin and rutin in water is sufficient to explain their relatively low activity by the oral route and the impossibility of giving them by injection, though slow absorption might well have some advantages in clinical use.

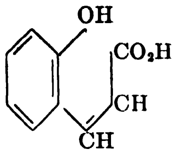
The first papers calling attention to the possible existence of a "Permeabilitäts-Vitamin" (an unfortunate description of substances that have no proven effect on capillary permeability) much confused the chemical picture for those who later took up the problem. The suggestion that "citrin," a water-soluble concentrate prepared from citrus peel, consisted of a mixture of hesperidin and demethylohesperidin, this being the 7-rhamnoglucoside of eriodictyol, had no experimental evidence to support it and was made more confusing by the proposal in one and the same paper that it should be called eriodictyn and eriodictyd (the latter subsequently explained in a personal communication as an uncorrected printer's error). This hypothesis failed to explain how removal of a single methyl group from hesperidin, which is virtually insoluble in water, could lead to a compound that was not merely water soluble, but hygroscopic. It is time, in our view, that references to eriodictyn, or demethylohesperidin, as having vitamin P activity and occurring in fruits showing that activity, should be avoided. The term "citrin," moreover, has—and always had—nothing more than a general descriptive significance, indicating the fruits of origin, and should not be used in any other sense than that of a citrus peel concentrate prepared by the method outlined in the first publication on the subject.

As has been recorded in published work, we have found citrus fruit concentrates having much greater activity than hesperidin, with which they were compared by the guinea pig capillary resistance technique. One was twenty times as active. It was completely soluble in water and could have contained no relevant quantities of insoluble compounds like hesperidin and rutin. The same is true of the single black-currant concentrate found to be about five times as active as the best citrus concentrates and thus one hundred times as active as recrystallized hesperidin.

More convincing is the evidence brought forward by French workers (Lavollay *et al.*, 1943) that the aglycone *d*-epicatechin which is very soluble in water, has high vitamin P activity: they refer to it as being at least 500 times more active than Szent-Györgyi's "citrin": the experimental findings leading to this conclusion are not given and one must accept this quantitative evaluation with reserve. Their method of testing the compound, which was probably contaminated with the normally occurring *d*-catechin, from which they prepared it by epimerization, was by intraperitoneal injection of an aqueous solution into normal guinea pigs. It is not possible, therefore, to evaluate it against hesperidin or rutin or any other substance tested only by the oral route on deficient animals.

These workers have naturally been led to suggest that traces of *d*-epicatechin may be present in even recrystallized hesperidin and rutin and could be responsible for the whole vitamin P activity of water-soluble citrus and black currant concentrates. Any decisions on this point must await further investigation, involving both an extension and an improvement of the biological test and a further study of the claims for a chemical test highly specific for *d*-epicatechin (Gero, 1946).

Javillier and Lavollay (1946) report that they have examined a number of compounds for vitamin P activity with a view to defining which parts of the molecule are essential to the activity. Without accepting many of the implications of their approach, which suggests that the physiological action of a molecule can be expressed as the sum of the activities of the chemical groupings present, we can agree that the activity of phloretol, which they assert to be great, is inconsistent with Szent-Györgyi's suggestion that the 3′,4′-diphenol structure is essential to a coupled oxidation-reduction system, possibly involving ascorbic acid, lying behind the physiological manifestation of vitamin P activity. The French workers are inclined to favor the view that the essential structure is



and thus that any compound having vitamin P activity without this arrangement must undergo metabolic changes leading to it. In our view, all such speculations are premature—at the very least.

The views just discussed have a certain similarity with those of Wawra and Webb (1942), who first suggested the chalcone theory of vitamin P activity, to which we have already alluded. Here also there is essential opening of the pyrone or pyran ring. Following up this suggestion, and in an attempt to prevent the rapid oxidation and recyclization characteristic of chalcones formed by heating on a boiling water bath in presence of normal alkali, C. W. Wilson ingeniously proposed to methylate the hydroxyl group formed during chalconization. It was hoped that this might give a stable compound, no longer susceptible either to ring closure or to oxidation under ordinary atmospheric conditions. A product prepared in this way was examined (Bacharach, 1945) and found to afford no evidence that it was a pure compound or even a mixture of pure hesperidin derivatives. It contained sodium chloride and other mineral matter contributing an "ash" content of 2% and the "corrected" Zeisel methoxy figure (20.65%) was over twice the theoretical one for monomethyl hesperidin chalcone (9.7%). It was no more active on guinea pigs (and not appreciably active on human subjects) than crude samples of unmethylated hesperidin chalcone and not more than five times as active as hesperidin itself. It cannot, therefore, have contained more than 5% of material as active as the richest black currant concentrate so far examined.

We are of opinion that no one has handled, let alone examined, specimens of pure hesperidin chalcone or hesperidin 1-monomethyl chalcone and that there is thus inadequate evidence for deciding whether it has a greater vitamin P activity, if any, than hesperidin itself. It does, however, appear that some substance or substances produced by the action of warm dilute alkali on pure hesperidin (possibly the chalcone) may have a greater activity than the flavanone glycoside itself. Conceivably some similar change might occur when ingested hesperidin (or rutin) reaches a portion of the gastrointestinal tract with contents at a suitable pH, but there is no information available on this point. Moreover, if the claims of Parrot and Lavollay are substantiated, it is difficult to see how chalconization can explain the rapid effect on the capillaries of normal guinea pigs caused by intraperitoneal injection of catechin.

In our view it is at present justifiable neither to attribute vitamin P activity to all aglycones and glycosides based upon the phenyl benzo-γ-pyran nucleus, nor to maintain that only substances based on that structure have vitamin P activity. For that reason, any attempt to tabulate here the physical properties and chemical reactions of all the recognized flavones and allied compounds would be as irrelevant as it would be irksone. Until there is clear-cut and incontrovertible evidence of the vitamin P activity of an unquestionably pure compound, natural or synthetic, the best we can do is to use as a base line of activity a series of highly purified samples of, say, hesperidin, in the hope that successive batches of such purified material will be identical in biological activity, within the limits of precision of the bioassay methods available.

III. Flavonoids from Plants

1. Occurrence

The flavanone glycoside hesperidin, first reported on by Lebreton in 1828, has now been found in most varieties of citrus fruit, although it is probably not present in either the grapefruit or the bitter Seville orange. It also occurs in many other plants, for example, in members of the Lobeliaceae, Valerianaceae, Umbelliferae, Labiatae, Compositae, Papilionaceae, and Rutaceae (for references see Higby, 1941). Highly active concentrates have been prepared from rose hips (see Wokes *et al.*, 1943) and from black currants (Pollard, 1942). Iwasaki (1936) examined the concentration of hesperidin in Mandarin oranges and reported 5.68% in the dried peel, 5.74% in imbedded fiber, 1.10% in the dried endocarp and only traces in the juice. Tseng and Yu (1936) reported 8.2% of pure hesperidin in dried orange peel and Harvey and Rygg (1936) found 3.07%. Many of these observations are based upon the finding of Pfeffer (1874) that hesperidin is deposited as sphero-crystals in the cells of sweet oranges by desiccation or by soaking the tissue in glycerol or alcohol. It is improbable, however, that this material is pure hesperidin. Oesterle and Wander (1925) found that many materials reported in the early literature to be hesperidin were in fact diosmin, and other compounds closely related to hesperidin also occur in citrus fruits—for example, neo-hesperidin in bitter oranges (Kolle and Gloppe, 1936) and naringenin in grapefruit (Asahina and Inubuse, 1929). How such a relatively insoluble substance as hesperidin is maintained in solution in the sap, or whether it is produced at the sites in which it is found, is unknown, but various theories concerning the formation of flavanones, flavones, and anthocyanins have been advanced (Robinson, 1936). It is regarded as established that such compounds are synthesized from carbohydrates, the reaction being favored by low temperature, light, and access to oxygen. Specifically, it is suggested that the flavanones are built up from two hexoses and one triose by means of aldol condensations, possibly through coniferyl alcohol as an intermediate. The flavonoids are believed to exist in plants predominantly as glycosides. Methods used by various workers for the extraction of hesperidin and other similar compounds are given by Higby (1941).

Lo (1942) examined eighty-three species of Chinese fruits and vegetables by the method of Lorenz and Arnold (1941). *Zizyphus vulgaris* Lam., variety inermis and *Rosa multiflora* Thumb., contained respectively 33.58 and 29.09 mg. of "vitamin P" per gram. Of seventy-six varieties of red peppers, six were richer in "vitamin P" than *Capsicum annuum* (Lo and Chang, 1942). Lo and Wu (1943) and Lo and Chen (1946a) report that the "vitamin P" content of vegetables as measured by the method of Lorenz and Arnold is increased by the addition of nickel, molybdenum or zinc to the soil.* Weatherby and Cheng (1943), using the chemical method of Wilson *et al.* (1942), found little or no flavone in cauliflower or lettuce. Much the highest amount was in lemon peel, 6.3 mg./g. dry weight, as against 0.27 for orange peel and 0.18 for grapefruit peel. Lorenz and Arnold (1941) reported 1.32–2.40 (mean 1.77) mg. "citrin" per gram of whole lemon, and Goldfarb *et al.* (1943), by their modification of the Lorenz and Arnold method, found that lemon juice contains about one sixth of the "citrin" (*sic*) content of cold aqueous extracts of lemon peel. Bacharach and Coates (1944) report that vitamin P as determined by biological assay in oranges and lemons is fairly uniformly distributed through the peel and the fruit. Although the methods used hitherto for the determination of vitamin P in plants, including that of Lorenz and Arnold (1941), have been highly unspecific and not very precise, it seems clear that flavones, flavanones, and similar compounds are widely distributed in the vegetable world and that they occur in surprisingly high concentrations in parts of certain plants, especially in the fruit and peel of lemons and oranges. A systematic study of a limited number of fruits, including orange and lemon, by valid biological and chemical methods should yield valuable results.

2. *Function in Plants*

The biological significance of hesperidin and other flavanones and flavones in plant metabolism is not in the least clear. Pfeffer (1874) reported the presence of hesperidin (*sic*) in the pitch and bark of young twigs, in leaves and in flower parts, as well as in the cells of the fruit flesh and pulp of the sweet orange. Rose petals were stated by Weatherby and Cheng (1943) to have a high content of flavones, the maximum value found being 4.4 mg./g. In *Rosa roxburghii* Franch., Lo and Chen (1945) reported that the "vitamin P" content increased with the size of the fruit, whereas the ascorbic acid content diminished. There was no correlation between the location on the branch and the content of either ascorbic acid or "vitamin P." Harvey and Rygg (1936) found, however, that the hesperidin (*sic*) content of the peel of Washington navel oranges was higher at the blossom end than near the stem end of the fruit. In Chinese lettuce, *Lactuca scariola L.*, the older leaves were richer in ascorbic acid but there was no correlation between "vitamin P" content and maturity (Lo and Chen, 1946b). Bruckner and Szent-Györgyi (1936) reported that unripe oranges contain large amounts of hesperidin but little eriodictyol glycoside which is, however, found in high concentration in the ripe fruit. They thought that "eriodictyol glycoside" was formed during ripening from hesperidin by demethylation. For reasons discussed above, the meaning of these statements is obscure. Harvey and Rygg (1936) found a decrease of from 3.07 to 2.03% in the hesperidin (*sic*) content of rind of Washington navel oranges during ripening. Lo and Chang (1942) report a rapid increase in the "vitamin P" content of Chinese peppers coincident with the change from green to red and also that the amount in legume seedlings increases during germination. The difficulty of evaluating the significance of this work emphasizes again the need for a more precise chemical method of estimation, upon the development of which largely depends any elucidation of the role of these materials in plant metabolism.

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* The justification for holding that Lo and his calleagues were dealing with vitamin P at all is questioned, by implication, earlier in the review.

That flavones and flavanones play an important part in the respiration of plants is suggested by the work of Huszák (1937a) and Szent-Györgyi (1937). Plant tissues can be divided into two large groups, containing either polyphenoloxidase or peroxidase, although, since both enzyme systems can occur together even in the same plant tissue, the separation between the groups is by no means sharp. The characteristic of the so-called polyphenoloxidase group is the brown discoloration which occurs on mechanical or chemical injury, as for example, in the banana, apple, and potato. Such plants contain catechol derivatives and polyphenoloxidases, which catalyze the oxidation of catechols to quinones. When the plant is damaged, the enzymes are released and there is a rapid formation of quinone, which combines with proteins and amino acids in the tissue with the production of dark brown pigments. In the peroxidase group of plants, however, there is no such formation of brown compounds (for example, the citrus fruits, cucumber, melon). This group is, however, characterized by the presence of peroxidases and of higher concentrations of ascorbic acid than the former group. As Szent-Györgyi (1931) originally showed, another enzyme, ascorbic acid oxidase (hexoxidase) is also present and is probably an integral part of the peroxidase system. Hydrogen peroxide added to the juices of peroxidase plants is quickly used up in the oxidation of their ascorbic acid, although purified peroxidase oxidizes ascorbic acid only very slowly. It has now been shown that the intermediate substance can be any one of a number of phenyl benzo-γ-pyrone pigments, provided that there are two adjacent hydroxyl groups in the phenolic nucleus, and presumably flavanones could act in the same manner. Although polyphenols can also function in this reaction, the pigments are some one hundred times more active. The reaction is not disturbed by catalase and can be mediated both by the glycosides and the aglycones. It may be summarized thus:

$$\text{Ascorbic acid} + O_2 \xrightarrow{\substack{\text{Ascorbic acid}\\ \text{oxidase}}} \text{dehydroascorbic acid} + H_2O_2$$

$$\text{Flavone} + H_2O_2 \xrightarrow{\text{Peroxidase}} \underset{\text{(quinonoid form)}}{\text{oxidized flavone}} + H_2O$$

$$\text{Oxidized flavone} + \text{ascorbic acid} \rightarrow \text{flavone} + \text{dehydroascorbic acid}$$

In this way all four valences of the oxygen molecule are employed in the oxidation of ascorbic acid, which can then be reduced again by means of dehydrogenases and sulfydryl compounds:

$$\text{Dehydroascorbic acid} + \text{glutathione} \rightarrow \text{ascorbic acid} + \text{oxidized glutathione}$$

$$\text{Oxidized glutathione} + \text{hexose phosphate} \rightarrow \text{glutathione} + CO_2 + H_2O$$

The phenyl benzo-γ-pyrone pigments can also be oxidized by polyphenoloxidases to colored compounds. Such reactions may account, in part, for the colored tissues of certain plants.

3. Function in Animals

The only clue to a possible role of the phenyl benzo-γ-pyrone pigments in animal metabolism is furnished by the work of Wawra and Webb (1942), who, by repeated precipitation of an aqueous extract of lemon peel with saturated $(NH_4)_2SO_4$ obtained a brown powder that, after exhaustive ether extraction, proved to be a protein-hesperidin chalcone complex. This complex was easily reduced by sodium hyposulfite and reoxidized by oxygen. Hesperidin chalcone was found to be autoxidizable, to diminish capillary fragility and to prevent "localized hemorrhages." It is reported that the protein and its prosthetic group, hesperidin chalcone, could serve as hydrogen carriers in mammalian liver in a system involving L-glutamic acid, apodehydrogenase, codehydrogenase I or II and diaphorase (Euler *et al.*, 1938). Wawra and Webb believe that the chalcone-protein and possibly similar substances might play a part in tissue respiration of both plant and animal cells. However, other reversibly oxidizable compounds can also play a similar role in this enzyme system so that this suggested natural functioning of the protein is not more than a possibility.

Another interesting observation was recently made by Bartlett (1948), who showed that synthetic 2′,3,4-trihydroxychalcone inhibits rat liver succinoxidase almost completely at pH 7.4 at a 1×10^{-4} molar concentration of the chalcone. Since there was no inhibition of cytochrome oxidase by this concentration of chalcone in a system containing catechol and washed rat-liver mince, it was concluded that the chalcone reacts with the substrate-activating dehydrogenase and not with the cytochrome C system. The inhibition can be prevented but not reversed by glutathione and the flavonoid is unable to prevent succinoxidase inhibition by cupric ions. Since the 3 methyl ether of the chalcone is inactive, the reaction is probably due to combination of quinonoid oxidation products of the chalcone with sulfydryl groups of the enzyme. The effect of this chalcone on capillary resistance is not reported. Hughes and Parkes (1946), however, synthesized 3,4-dihydroxychalcone and its 3-methyl ether and found both to elevate capillary resistance in guinea pigs (Bourne's method). Inhibition of urease and succinoxidase by quinonoid oxidation products of *p*-aminophenol and *p*-phenylenediamine and by a large number of compounds whose only common denominator appears to be their ability to react with SH-groups has, however, been described by Potter (1942) and by Potter and DuBois (1943), so that there is no reason to suppose that this property is specific to the flavonoid compounds.

Finally, it should be mentioned that Karrer and Viscontini (1947), studying a system containing 5 mg. pyruvic acid in 0.3 ml. water and 100 mg. of dried yeast in 2 ml. of phosphate buffer at pH 6.2, have reported that cocarboxylase is inhibited by flavonols. However, this action is shared by a number of substances of varying chemical nature, for example, patulin, β-ionone, acetophenone, phenol, acetaldehyde and acrolein.

IV. PHYSIOLOGICAL ACTION OF VITAMIN P

1. *Effect on the Cardio-Vascular System*

Among the first to study the biological actions of flavonoids in animals was Akamatsu (1931), who reported that quercetin, quercitrin, rutin, myricetin, and myricitrin increased the stroke volume of both the intact and the isolated frog's heart. The heart rate slowed and the minute volume increased. These observations were confirmed by Fukuda

(1932); von Jency and Czimmer (1936) and Sokoray and Czimmer (1938) reported that quercetin, quercitrin, and rhamnetin "increased the function" of the intact heart and could restore the function of hearts poisoned with chloroform, urethane or lactic acid. Since hesperetin was inactive or actually toxic, they concluded that activity requires two hydroxyl groups in the phenolic nucleus as previously claimed by Bentsath *et al.* (1937) for modifying the course of scurvy in guinea pigs.

Jeney *et al.* (1937) later described a fall of blood pressure following injections of quercetin or quercitrin. The latter phenomenon was studied in more detail by Armentano (1938a), who injected both dogs and cats intravenously with solutions of purified materials. Hesperidin, hesperetin, eriodictyol and homo-eriodictyol were found to be inactive. Quercitrin, quercetin, citrin, naringenin, rhamnetin and 6:8:-hydroxyflavone produced more or less marked falls in arterial pressure. In dogs tachyphylaxis was produced in 60% of the animals, but not in cats. It was considered that the fall in pressure was due to a fall in peripheral resistance rather than to impaired cardiac output. These observations led Armentano to the conclusion that "citrin" prepared from lemons by Szent-Györgyi's method must contain some flavonoid material other than hesperidin and eriodictyol. Lesser *et al.* (1941) studied the effects of an aqueous solution of a crude extract of lemon peel on the vascular system. A fall of blood pressure in the cat and rabbit followed its intravenous injection even after section of both vagi. Complete atropinization, however, abolished it in the cat. The isolated frog heart was slowed and "its systolic contractions were decreased." Higby (1943) also reported that purified hesperidin and eriodictyol were without effect on arterial pressure, while crude hesperidin was depressor. He considered this to be due to the presence of hesperidin in the form of its chalcone. It seems to be established that flavonoids may produce a fall in arterial pressure in animals, but there is at present no reason to think that this is associated with any effect on capillary resistance. Armentano (1938) described general malaise, fever, and dyspnea following the intravenous administration of quercitrin and rhamnetin to man, although previous injection into animals had been without untoward effect, and Raunert (1938) mentioned that blood pressure was reduced by 10 to 15 mm. Hg after a single intravenous injection of 55 mg. of "citrin." We have never observed any ill effects to follow injection of vitamin P preparations in man, but such injections should be made with extreme care.

It may be recorded here that some pharmacological studies of our own (unpublished) showed that "methylated hesperidin chalcone" (discussed above) was capable of producing in rabbits a striking depression in blood pressure and maintaining it for a long time—longer than any other substance known to us—without killing the animal. The effect is potentiated by atropine, even if this is administered some time after the chalcone's effect has fallen off. There is also evidence to suggest that the site of this depressor action is the heart and not the peripheral vessels. Our doubts as to the bearing of any of these facts on "the vitamin P problem" arise first from the fact that the chemical nature of the methylated chalcone (see above) is far from unambiguous and, secondly, because a water-soluble citrus concentrate used in the laboratory as a standard preparation for the bioassay of vitamin P was found (though admittedly it had been stored for nearly two years without reconfirmation of its activity) to have no appreciable effect of any kind on the blood pressure of the rabbit.

The phenomenon so far most closely associated, in both animals and man, with vitamin P, namely, a change in capillary resistance, has already been discussed in some detail. Elevation of capillary resistance has been reported to follow oral, subcutaneous, intramuscular, intraperitoneal and intravenous administration. Almost nothing is known about how this effect is exerted, but Scarborough (1942b) has briefly reported preliminary experiments which suggested that the action was not a direct one on capillary walls. He attempted to introduce a water-soluble vitamin P preparation through the skin from a lanoline base, but found no elevation of capillary resistance at the site through which the preparation had supposedly been introduced. That sufficient material to affect capillary resistance can be introduced in this way was shown by anointing the skin with a similar lanoline base containing soap. This produced a sharp fall in capillary resistance in the area treated. The work of Lavollay and others, which is further discussed below, has been taken to suggest that elevation of capillary resistance is produced by adrenaline and that vitamin P functions by protecting adrenaline from oxidation, raising capillary resistance indirectly.

Another interesting line of study has been opened up by the work of Beiler and Martin (1947, 1948), who suggest that vitamin P may increase capillary resistance by inhibiting hyaluronidase. They were able to show by an *in vitro* test that the inhibiting effect of ascorbic acid at a concentration of 0.1 mg./ml. was potentiated by purified hesperidin and its methylated chalcone and by rutin at a similar concentration. Only rutin inhibited hyaluronidase in the absence of ascorbic acid and not below the relatively high concentration of 1 mg./ml.; esculin, its aglycone, and 4-methylesculetin were also active. Acetylated, sulfonated or phosphorylated hesperidin produced marked inhibition of hyaluronidase at a concentration of 0.1 mg./ml. in the absence of ascorbic acid. If hyaluronic acid were the substance maintaining the integrity of capillary walls, then hyaluronidase activity might lead to increased fragility and inhibition of the enzyme to a restoration of their resistance.

Raunert (1938) considered that vitamin P ("solubilized citrin") controlled hemorrhage only when the serum calcium was raised by the injection. We have not been able to confirm this. A rather extensive study (unpublished) of blood calcium levels in scurvy and other vitamin deficiency states, their relation to the subcutaneous bleeding and the effects of ascorbic acid and vitamin P preparations upon them does not suggest any connection between serum calcium and either the capillary resistance or the occurrence of bleeding.

In man the capillary resistance is at its highest value some 24 hours after the administration of a large dose of a vitamin P preparation. At 12 hours some increase is apparent, while 48 hours after administration the capillary resistance has usually returned to its initial level. Following a single intraperitoneal injection of epicatechin into a guinea pig, Parrot *et al.* (1944) describe a conspicuous increase in capillary resistance; it begins in half an hour and lasts 4-5 days. It is remarkable that the elevation of capillary resistance in man follows apparently the same course after both oral and parenteral (even intravenous) administration, and that the responses to the oral administration of such a relatively insoluble substance as hesperidin are so uniform as to make it possible to construct a dose response curve from them (Scarborough and Wokes, 1947).

An important contribution to our knowledge of the actions of vitamin P has been made by a group of French workers. Lavollay (1941) concluded that although flavones and related compounds had no direct effect on isolated strips of guinea pig intestine, they were able to prolong the effects of adrenaline, presumably by protecting this from oxidation (Lavollay, 1942). Lavollay and Neumann (1941) reported that quercitrin would prolong the action of adrenaline on the blood pressure of dogs, and Parrot (1943) obtained prolonged adrenaline contraction of the nictitating membrane of the cat after treatment with epicatechin. Parrot and Lavollay (1944a) studied the contraction of the cat's nictitating membrane after intravenous adrenaline. They found that the contractions were increased and lasted much long if epicatechin (as an extract of *Acacia catechu*) was given also. These observations receive confirmation from the studies of Wilson *et al.* (1947), who showed that the action of adrenaline on strips of guinea pig intestine was prolonged by the addition of rutin to the bath, the time of prolongation of the adrenaline relaxation being directly proportional to the dose of rutin. Concentrations of rutin below 0.2 mg./50 ml. frequently caused relaxation directly. Quercetin was much more potent than rutin in this respect and the phenomenon was attributed to protection of adrenaline from oxidation. Parrot and Lavollay (1944b) gave adrenaline intraperitoneally to guinea pigs and intramuscularly to man and showed that capillary resistance was elevated for several hours. Parrot *et al.* (1944) then studied the effect on capillary resistance of 40–120 μg. of adrenaline given intraperitoneally to three guinea pigs on a diet low in greenstuffs. Following a single intraperitoneal injection of 1 mg. epicatechin, the same amount of adrenaline a few days later produced on capillary resistance an effect four times as great and lasting three times as long. Similar results were obtained in a young man. These results find confirmation in the report of Berez *et al.* (1948) that intravenously administered rutin causes a 2.5 fold increase in the sensitivity of vessels in the rat meso-appendix to topically applied adrenaline. Parrot *et al.* (1944) showed that the capillary resistance of normal and scorbutic guinea pigs and of a healthy man of 65 with fragile capillaries was greatly increased by the parenteral administration of a mixture of catechins containing *d*-epicatechin. The ability of mixed natural epimers of catechin to increase capillary resistance was evidently proportional to its content of epicatechin. Lavollay (1944) now considers, therefore, that the role played by vitamin P in increasing capillary resistance is related to its ability to inhibit the oxidation of adrenaline *in vivo*. The capillary resistance of normal guinea pigs was elevated by other materials capable of inhibiting the oxidation of adrenaline, including ascorbic acid. A summary of the work and general conclusions of the French group is given by Javillier and Lavollay (1946), who restate their concept that substances possessing vitamin P activity function through their ability to delay the oxidation of adrenaline *in vivo*. Adrenaline itself is the cause of the elevated capillary resistance. They question the use of the term vitamin P on the grounds that there are so many chemical compounds of unrelated structure which possess this property. Lavollay (1945) had already suggested that the term vitamin P_1 be used for compounds with a phenylchroman structure and vitamin P_2 for those derived from benzpyrone.

A recent paper by Clark and Geissman (1949) follows the lead of the French workers and records a detailed study of flavonoids as adrenaline potentiators. They used rutin as "standard" and found approximately equal activity in about 17 compounds and greater activity in about 15 others, of which not all were flavonoids. A considerable number showed no activity at all. These workers hold that vitamin P activity cannot be attributed to a delay in the oxidation of adrenaline or sympathin.

In another important communication Haley *et al.* (1947) have described the topical application of a number of purified materials to the rat meso-appendix preparation of Chambers and Zweifach (1944) and observation of the behavior of the capillary bed under the microscope in a total of seventy-six animals. The relative potency of various compounds in promoting constriction of (presumably) the precapillary sphincters was determined by finding the dilution at which this effect could still be observed. Rutin and its sodium acid succinate, hesperidin, its sodium acid succinate and its methylated chalcone, esculin and adrenochrome were found to be inert even at concentrations of 100 μg./0.1 ml. The inactivity of rutin topically applied was ascribed to its relative insolubility; this is interesting in view of the statement of Berez *et al.* (1948), mentioned earlier. The sodium acid phthalates of both rutin and hesperidin produced slight vasoconstriction at dilutions of 100 to 10 μg./0.1 ml. but *d*-catechin, epimerized *d*-catechin and *d*-epicatechin were extremely active, the last at 1×10^{-7} μg./0.1 ml. This finding is in agreement with Parrot (personal communication), who regards *d*-epicatechin as the most active vitamin P compound so far studied. Haley *et al.* (1947) also found that decomposition (perhaps oxidation) products of catechins, "catechin red," induced irreversible vasodilatation and stasis. They regard such materials as endothelial poisons and state that marked hemorrhagic symptoms follow their intravenous injection. Previously Parrot and Cotereau (1945) had described a fall in capillary resistance following administration of similar materials to which they unhappily gave the collective name "antivitamin P." They also describe a fall in capillary resistance following the intraperitoneal injection into guinea pigs of 10 μg. of an alcoholic extract of turnips. If such material really exists in foodstuffs, it might be responsible for some of the conflicting views on the effect of dietary variations on capillary resistance. Bacharach and Coates (1943), however, found appreciable vitamin P activity in turnips.

It is interesting to consider the observations of Haley *et al.* (1947) alongside those of Fuhrman and Crismon (1948), who studied the effect of the oral administration of rutin in daily doses of 50–100 mg./kg. for various periods of time on the course of experimental frostbite in rabbits, for the production of which a standard technique had previously been elaborated. Fuhrman and Crismon's careful observations led them to conclude that animals treated with rutin suffered considerably less tissue destruction than controls when the injury involved the feet, although rutin was ineffective in preventing loss of tissue in the ears. The time required for the development of wet gangrene in treated animals was significantly longer than in a control group ($P < 0.001$) and the area involved was much smaller. Direct observation of the circulation in the minute vessels of the rabbit's ear after thawing of the part showed that stasis, presumably dependent on loss of fluid from damaged capillaries, was delayed up to 80 minutes in rutin-treated animals, whereas it was normally complete within 10 minutes in controls. Parallel with this finding was the observation that the time of appearance of intravenously injected trypan blue in frozen areas of the abdominal skin of rats was approximately twice as long in treated animals as in controls. Somewhat similar observations on the effect of rutin in delaying the appearance of trypan blue in injured areas of skin are reported by Ambrose and DeEds (1947). From direct observations of the circulation (Crismon and Fuhrman, 1947a) and indirect evidence based upon the appearance in the tissues of intravenously injected fluorescein (Crismon and Fuhrman, 1947b), the authors conclude that a retarded rate of protein loss into tissues injured by excessive cold depends upon reduction in the total area available for filtration rather than on decreased capillary permeability. For these reasons and because the effect of rutin on the escape of trypan blue into frozen areas was not reproduced by privine, which constricts arterioles, Fuhrman and Crismon believe that alterations in the pattern of blood flow through the capillary bed followed the administration of rutin and was the basis for their striking findings. Occlusion of capillaries by masses of packed red cells and by contraction of precapillary sphincters retards or halts the flow of blood through the minute vessels so that the loss of protein-containing fluid from them is much retarded.

These two papers are important for the light they throw on a possible mechanism of action of vitamin P, although it must be remarked that the doses used by Fuhrman and Crismon were relatively large. That constriction of metarterioles and precapillary sphincters follows oral administration of rutin and the topical application of certain soluble flavonoids appears to have been established. This reduces or even arrests the flow of blood through capillary beds. Vasoconstriction, evidently arteriolar, had been described before in both amphibian and mammalian extremities perfused with quercetin, quercitrin, quercetin diglycoside, rutin or myricetin. Whether these phenomena are due to a direct action of the preparation on vessels or whether they are mediated through circulating endogenous adrenaline are questions which remain to be answered. The observations are of great interest in relation to the work of Lee and Lee (1947), which seems to demonstrate a pronounced loss of sensitivity to topically applied adrenaline in the minute vessels of the exposed mesentery of unanesthetized, scorbutic guinea pigs. This phenomenon was confined to vessels distal to terminal arterioles and, together with dilated venules, absence of vasomotion and an increased tendency to petechia formation, was attributed specifically to lack of ascorbic acid because none of these things were ever observed in a control group of animals on the same diet plus 15 mg./100 g. of body weight per day of synthetic ascorbic acid. Here it is pertinent to point to the hypertrophy of the adrenal glands in scorbutic guinea pigs, reported by Quick (1933), Hou (1934) and Todhunter *et al.* (1940). Any final statement as to relative effects of ascorbic acid and "vitamin P" in maintaining or restoring the sensitivity of minute vessels to adrenaline must await the outcome of further work. At present one can do no more than comment that Lee and Lee's experiments appear to have been most carefully designed and controlled but that all their diets contained 5% yeast. If further nutritional studies are to be made in animals, someone must determine whether yeast may contain "vitamin P."

The studies outlined above also draw attention to the possibility that the apparent increase in capillary resistance in the skin following the administration of hesperidin may be due to reduced blood flow through cutaneous capillaries rather than to a real increase in the resistance of capillary walls to the application of stress. One of the most serious objections to capillary resistance tests is that it is impossible to say what exactly is happening to the minute vessels during the test and thus to determine the mechanism of petechia production. Experiments to eliminate these objections will not be easy either to devise or to interpret, but they might be expected to elucidate some of the reasons why capillary resistance tests apparently work in certain laboratories and not in others.

In the matter of adrenaline potentiation it appears to have been established that both phenylchroman and benzpyrone compounds can prolong the action of adrenaline on the nictitating membrane and the isolated intestinal strip. It is reported also that the vessels of the rat meso-appendix are rendered much more sensitive to topically applied adrenaline by the intravenous administration of rutin. It is, however, doubtful if this phenomenon can explain the effect of vitamin P on cutaneous capillary resistance as suggested by Lavollay (1944). It is true that subcutaneous injection of adrenaline may be followed by an apparent increase in capillary resistance in man, but in our experience this increase occurs only after administering doses of adrenaline large enough to alter blood pressure and heart rate and to blanch the skin. The effect is observed during some 20–90 minutes following the injection and could be interpreted as evidence that cutaneous vasoconstriction is the cause of the apparent increase in capillary resistance. However, the elevation of capillary resistance after hesperidin is given by mouth is not attended by any obvious disturbance of the circulation either generally or in the skin and the effect is maximal some 24 hours after administration. These remarks apply also to parenteral administration of water-soluble vitamin P preparations.

Finally, it is necessary to refer to the effect of flavonoid compounds on histamine sensitivity and shock. Hiramatsu (1941b) reported that vitamin P (hesperidin) protected guinea pigs from anaphylactic shock, and Lavollay and Neumann (1941) stated that the anaesthetized dog was protected by quercitrin from the fall in blood pressure normally produced by peptone shock. Parrot and Richet (1946) studied the effects of intraperitoneal injection of histamine on guinea pigs. Histamine sensitivity was increased on a scorbutogenic diet, but was reduced to normal either by ascorbic acid or by "vitamin P." They made the interesting observation that the animals with the highest capillary resistance were not necessarily those least sensitive to histamine. Wilson *et al.* (1947) extended these observations and showed that the intraperitoneal injection of rutin in guinea pigs decreased the mortality produced by approximately LD_{50} of histamine if the rutin injection preceded the histamine injection by 10–30 minutes. The findings that protection was not afforded by simultaneous injection of the two drugs and that the action of histamine on perfused isolated bronchi and on strips of guinea pig colon was not altered by rutin suggested that the protective effect of rutin was indirectly produced. It was postulated that the rutin retarded destruction of adrenaline and, therefore, resulted in a slightly increased level of adrenaline in the blood. In a recent communication Roth and Shepperd (1948) reported failure to protect guinea pigs from the anaphylactic shock following sensitization with horse serum or with egg white-Freund adjuvant, when they used "protective" doses of rutin ranging from 1 to 20 mg. Nor could they counteract the effect of histamine shock. We would also refer here to some (unpublished) findings of our own. We administered to guinea pigs the water-soluble citrus concentrate already tested for its effects on the rabbits' blood pressure (see above). Some animals were sensitized to casein before and others after the vitamin P treatment. Sensitivity to the casein was established either by a second "shock" dose of casein or by the Dale uterus technique. No effect of the vitamin P treatment could be demonstrated.

Perhaps this is the place to recall Borbély's (1930) observation that injection of salvarsan sometimes increased capillary fragility and that Horne and Scarborough (1940) reported a uniformly low capillary resistance in ten individuals with toxic erythema or dermatitis developed in the course of antisyphilitic therapy with arsenic and bismuth. Horne and Scarborough subsequently reported a second series (1941) of nine similar cases in which capillary resistance was uniformly low; in some of them it was found that the fall in capillary resistance preceded the appearance of clinical signs and symptoms. They suggested, therefore, that regular determinations of capillary resistance might help to prevent the occurrence of toxic effects in patients having antisyphilitic or other treatment with heavy metal compounds. Horne and Scarborough (1940) confirmed the report of Scarborough and Stewart (1938) that vitamin P preparations can increase capillary resistance in these toxic states. They found that ascorbic acid was ineffective. Gorrie (1940) has also reported that hesperidin is effective in the treatment of arsenical purpura. Goldstein *et al.* (1943) found that treatment with hesperidin methyl chalcone reduced the mortality caused by a standard dose of mapharsen in rabbits from 90 to 57%, a difference of doubtful significance ($\chi^2 = 2.9$), and Friend and Ivy (1948) reported a favorable but not statistically significant effect on the survival rate of mice given injections of dichlorophenarsine hydrochloride. A protective action of ascorbic acid against arsenical toxicity has been noted by several groups of workers (Sulzberger and Oser, 1935; Bundesen *et al.*, 1941; McChesney *et al.* 1944, and others). Friend and Ivy found that ascorbic acid, like "hesperidin methyl chalcone," had a favorable but not significant effect in mice against dichlorophenarsine poisoning. When ascorbic acid and "hesperidin methyl chalcone" (47.6 mg./kg./day) were administered together, the protection afforded against a single toxic dose of dichlorophenarsine was highly significant ($\chi^2 = 70.6$, 111 mice in control group, 112 in treated group).

It is interesting to speculate upon a possible connection between these observations and those on histamine sensitivity discussed above. Lewis (1927) believed that many toxic skin reactions are the result of the liberation of H-substance by damaged tissue. He has presented evidence that H-substance has many of the properties of histamine. Such skin reactions (toxic erythema) are characteristically associated with lowered capillary resistance and are not infrequently alleviated by the administration of adrenaline. They have been reported to be relieved by preparations of vitamin P, which produce an associated increase in capillary strength. It seems possible that this effect of vitamin P might be the result of an adrenaline-protecting action, following which sensitivity to histamine becomes reduced.

2. *Other Physiological Actions*

Euler and Malmberg (1937 and 1938a) reported that the administration of either ascorbic acid or "citrin" to guinea pigs which had been receiving a scorbutogenic diet for about 25 days increased the number of circulating reticulocytes until, in some cases, they comprised 30% of the total red cells. A similar effect was observed after injections of adrenaline, acetylcholine or an extract of garlic (1938b), but was not found in guinea pigs on a normal diet. According to Armentano (1938b), however, scurvy in guinea pigs is frequently associated with anemia characterized by the appearance of reticulocytes in the peripheral blood, and this is also true in man (Dunlop and Scarborough, 1935). The anemia and reticulocytosis could be prevented by ascorbic acid but not by subcutaneous injection of "citrin." The number of reticulocytes in the peripheral blood of fasting normal subjects was not increased by either ascorbic acid or "vitamin P" (Müller, 1939). Neither the total nor the differential leucocyte count was significantly altered by the administration of "vitamin P" to fasting control subjects or patients suffering from various diseases, though it was followed by slight increases in the platelet count (Müller, 1939). A marked shortening of the bleeding time was reported to follow the intraperitoneal injection into guinea pigs of a mixture of epimers of *d*-catechol, and similar results were obtained when this preparation was given subcutaneously to men with abnormally prolonged bleeding time (Parrot and Galmiche, 1945). These results have been confirmed by Ungar (1945). Vacek (1941), however, could find no constant effect of "citrin" on bleeding time in man.

A recent brief report (Griffith *et al.*, 1947) suggests that capillary resistance in the peritoneum of rats is markedly reduced 1 to 8 weeks after irradiation from a radon ointment, and that in rats the administration of rutin (20 mg. every third day for 36 days parenterally) accelerates recovery from X-ray injury. Rekers and Field (1948) also report that rutin in doses of 50 mg. three times a day by mouth for 1 week prior to and during X-irradiation significantly increases the survival rate of dogs submitted to total body irradiation of an approximately "mid-lethal dose" (χ^2 = approximately 20). The amount of bleeding, associated with thrombocytopenia, in the treated group was much less than in the controls, although there was little difference in hematological findings. This is at variance with all the clinical reports on the effect of "vitamin P" in idiopathic thrombocytopenic purpura.

The effect of ascorbic acid in promoting wound healing (Hartzell and Stone, 1942) is independent of vitamin P, and pure synthetic ascorbic acid by itself is capable of accelerating the deposition of calcium salts in normal and regenerating bone (Bourne, 1942 and 1943a).

Two other biological effects claimed for vitamin P should be mentioned. Following the observations by McCarrison (1920) of increased activity of the thyroid gland in scorbutic guinea pigs, Cotereau *et al.* (1946) reported appreciable activation of the gland in male guinea pigs on a scorbutogenic diet supplemented with 2 mg. ascorbic acid daily. They also reported (Gabe *et al.*, 1947) that subcutaneous injections of a mixture of epimers of *d*-catechol caused the thyroid glands of normally fed guinea pigs to enter the resting phase. Lecoq *et al.* (1947) reported an increase in muscular and a decrease in nervous chronaxie in guinea pigs after 3 to 5 days on a scorbutogenic diet. Administration of "vitamin P" delayed the onset of these changes for 8 to 11 days. A similar effect was, however, exerted by a number of other substances, including adrenal cortex.

3. *Intermediary Metabolism of Flavonoids*

Very little is known of the fate of flavonoids in the animal body. The main reason for this is the lack of a satisfactory method for their chemical estimation. Fukuda (1932) reported that 50 to 90% of the intravenously injected flavonol glycoside quercitrin was excreted by the kidneys and 30 to 70% by the liver into the bile. In a more detailed study Huszák (1937b) attempted to investigate the fate of parenterally injected "citrin" solutions (flavanone glycosides) in rabbits, using three color reactions, viz., the cyanidin reaction, the red color in alkali and the reaction with ferric chloride. By the methods employed he was able quantitatively to recover "citrin" added to various tissues. Following the intravenous injection of 100 mg./kg. of "citrin," 40 to 60% was excreted within 1 hour and after 18 hours only traces remained. In the first 10 to 60 minutes following injection, the flavanone content of the kidney was approximately double that of the liver, weight for weight. One hour later, however, the liver contained more than the kidney and 20 hours after the injection about 20% of the flavanone was found in the bile. Intestinal wall, muscle, skin, blood vessels and brain contained very little flavanone at any time. After 36 hours there was no difference in the amounts of flavanone in the tissues of injected and control rabbits, neither was the total body flavanone content of injected white mice different from that of controls. If the method can be depended on (and our own experience with these color reactions has not been at all encouraging) these results are important as indicating that only traces of injected flavanone ("citrin") are taken up by the tissues, the collected excreta containing almost as much as was injected. Huszák (1937b) also reported that continued daily injections led only to insignificant increases in tissue concentration. It is impossible, at present, to evaluate the significance of these results, but it is difficult to see how they fit in with the findings of Parrot *et al.* (1944) that the capillary resistance of guinea pigs after a single injection of epicatechin remains elevated for 4 or 5 days. We have always found that the capillary resistance has returned to its initial value some 36 to 48 hours after a single dose of flavanone whether given by mouth or parenterally. Armentano *et al.* (1938) attempted to measure the "citrin" content of human urine by using the silver lactate method referred to below (p. 43). They reported that "citrin" is excreted in normal urine to the extent of 11 to 30 mg./day and that administration of "citrin" or eriodictyol to apparently healthy individuals produces saturation in 2 to 6 days with an excretion of 50 to 100 mg./day. Although in thrombocytopenic purpura saturation occurred normally, in vascular purpura saturation could not be achieved even by administration over long periods. Confirmation of these results would be important evidence for the vitamin nature of flavanones and might also shed light on vascular purpura; this would then be revealed as a vascular disorder in which, for some presumably metabolic reason, the requirements for the vitamin are greatly increased. Our own attempts to use this chemical method for the estimation of flavanones in urine have met with no success at all.

It is unfortunate that the uncertainty of chemical methods for the determination of flavanones and similar compounds in biological material has so far precluded any precise study of the absorption and excretion of these compounds. It is reported by Kirtley and Peck (1948) that even very large doses (15 g./day) of "hesperidin methyl chalcone" given to patients are not followed by any increase in urinary excretion as estimated by the procedure of Wilson *et al.* (1942). However, Garino (1913) gave hesperidin both intraperitoneally and intravenously to dogs and isolated hesperidin, but not hesperetin, from the urine. In a single dog he was able to recover 1 g. of unchanged hesperidin from the urine after the injection of 1.5 g. of the material. Intravenous hesperetin was also excreted unchanged. Ambrose and DeEds (1947) examined the urine of rabbits and of dogs given rutin intravenously and the urine of a man who received 1 g. by mouth and reported that rutin was excreted rapidly (method unstated). Scarborough (unpublished) noted a conspicuous increase in ethereal sulfate excretion in the urine when 0.5 g. of hesperidin was given by mouth to three patients for 5 days. It seems likely, therefore, that flavonoid compounds, although relatively insoluble in water, are readily excreted even after oral administration. Studies are required of the fecal and urinary contents of flavonoids during and between periods of oral administration of pure preparations that can be shown to elevate capillary resistance. In this connection one recalls the remarkable regularity of response to the oral administration of the almost insoluble hesperidin and the fact that biological assays of this material in both guinea pigs and man against the provisional standard of Bacharach and Coates (1942) regularly gave almost identical results (100 provisional units per gram).

4. Toxicity of Flavonoids

With the exception of the disturbances of arterial pressure to which we have already referred there is little evidence that the administration of flavonoids to animals or man is attended by any untoward effects.

Wilson and DeEds (1940) fed young healthy male albino rats for 200 days on a standard laboratory diet containing either naringin or hesperidin in concentrations as high as 1% by weight of the diet, but could observe no evidence of cumulative injury as judged by growth curves, weights of important organs (heart, liver, spleen, adrenals, kidneys and testes) and macroscopic and microscopic examinations of tissues. Wilson, Mortarotti and Doxtader (1947) found no acute or chronic toxicity with large doses of rutin. It must be remembered also that these compounds are so widely distributed in plants that the daily intake of many persons may be considerable. We have often given impure hesperidin by mouth in doses of 1 g. per day to patients for as long as 30 days with no sign of any ill effect, nor have we ever observed any toxic reaction, similar to those described by Armentano and referred to previously, following the parenteral (including intravenous) administration of active soluble materials.

Kirtley and Peck (1948) gave as much as 15 g. daily by mouth of the water soluble "hesperidin methyl chalcone" for an unstated time without observing any ill effect. They were, however, unable to detect the compound in the urine so that, as in the other studies referred to above, the possibility arises that the material was incompletely absorbed.

V. Capillary Resistance in Non-Scorbutic Disease

There are many published reports that seem to demonstrate the value of vitamin P in a wide variety of diseases, but much of this work must be discredited because of the lack of adequate control periods, or control groups not receiving the vitamin, insufficient numbers of cases, absence of statistical evaluation of results, unreliable methods for assessing capillary resistance and too infrequent determinations, and the unsatisfactory nature of some of the preparations administered. However, it does seem to be clear that vitamin P has elevated capillary resistance in many individuals with apparently quite unrelated clinical conditions. There is no evidence that the majority of such patients were living on diets that might be deficient in the vitamin, but one could suppose that their requirements for vitamin P were greater than normal as the result of the disease. However, this and much else is speculation and we hold, therefore, that it would serve little purpose at the present time to review the clinical studies in detail; suffice it to say that there is at present apparently no clear indication for the administration of vitamin P. It is necessary to reiterate, moreover, that it has still to be shown that an abnormally low capillary resistance itself confers any disability on the subject. It is also true that there has not so far been readily available for clinical use a pure, stable, soluble, reliable and highly potent preparation. In its absence caution is needed in evaluating the results of treatment with materials possessing only some of these desired characteristics.

Whatever may be the final conclusion as to the efficacy of vitamin P, either in elevating capillary resistance or in ameliorating the clinical condition, a considerable experience in the clinic with capillary resistance tests of various kinds, as well as a study of the literature, indicates that there are three disorders in which unduly fragile capillaries are commonly met with. In these conditions a low capillary resistance usually persists at least for many weeks and, so far as present experience goes, is not subject to sudden changes. The most striking of these is *thrombocytopenic purpura,* in which the capillary resistance as determined by negative pressure methods is consistently very low. It is probably not going too far to say that this is the only disease in which abnormally fragile capillaries are invariably found. It is now generally agreed that the preparations of vitamin P that have hitherto been available neither elevate capillary resistance nor increase the thrombocyte count in this condition (Armentano *et al.*, 1936; Vaughan, 1937; Scarborough, 1943; Rudy *et al.*, 1944). In cases of *diabetes mellitus* Armentano *et al.* (1936) found abnormally fragile capillaries by Borbély's test, Conn (1945) reported low capillary resistance in 30 of 120 diabetic patients and Rodriguez and Root (1948) in 40% of 100 unselected diabetic subjects (Göthlin's test). Beaser *et. al.* and Rudy *et al.* (1944) also found abnormal capillary resistance in diabetes (Wright's test). Our own experience with a negative pressure test is similar. The third condition of which unduly fragile capillaries are often a feature is *essential hypertension.* In 18% of 265 selected cases, Griffith and Lindauer (1944) found abnormal fragility by Göthlin's test (see also Griffith *et al.*, 1944). Similar findings were reported by Shanno (1946) and are confirmed by Scarborough (unpublished). The report of diminished resistance in hypertension by Beaser *et al.* (1944) cannot, however, be admitted because Wright's method was used for assessing capillary strength. In this procedure the capillaries in hypertensive subjects are submitted to much greater stresses than are the capillaries of persons with lower arterial pressures, and this makes the results difficult to evaluate. The recorded results of vitamin P therapy on the abnormally low capillary resistance in diabetes mellitus and hypertension are conflicting.

VI. METHODS OF ESTIMATING VITAMIN P

1. *Biological Assay*

Many of the figures for "vitamin P content" to be found in the widely scattered but already considerable literature have perforce to be ignored. Sometimes they are based on color or other chemical tests that may (or may not) be specific for particular compounds or structures related to 2-phenyl-1:4-benzpyrone (formula I); sometimes the authors do not explain the basis for their statements.

In our view the state of our knowledge about vitamin P, in particular about its physiological and pharmacological properties and about the chemical structure of compounds showing these properties, demands critical restraint in making claims for vitamin P activity. The final appeal test is the biological one, as it always has been and must be for all substances acceptable into the group of vitamins. Chemical or physicochemical tests can only be validated after many applications have shown that they have a high and specific degree of correlation with the appeal test on deficient animals (or human patients). Not only must the vitamin in question (and the more necessarily if it exists in more than one chemical form) invariably give in the chemical test positive results that are quantitatively related to the biological activity, but no inactive substances likely to be encountered when estimating the vitamin must act to the test even at relatively low concentrations. These considerations may seem elementary: our excuse for restating them is that they do not always seem to have been borne in mind, especially during the relatively brief life-history of vitamin P.

Now this substance was first defined—and, as we think, wrongly defined—in terms of its action on capillary *permeability*. It seems justifiable, all the same, to insist that prophylactic or curative action on capillary *fragility*, that is, the maintenance or restoration of capillary resistance, in animals (or men) with fragility increased because of vitamin P shortage (from inadequate or ill-utilized food supplies) is the test and the only decisive test of the presence of vitamin P. For assay purposes, this demonstration was first made by Zacho (1939) on guinea pigs receiving a scorbutogenic diet and pure vitamin C (see Section III, 1). His method was studied and developed by one of us and his colleagues (Bacharach *et al.*, 1942; Bacharach and Coates, 1942), while the other of us extensively studied the effect on human subjects of those substances giving positive effects on guinea pigs. The basis of the assay method and the details of its application were described in two papers mentioned above. Two subsequent papers (Bacharach and Coates, 1943, 1944) gave results for various British fruits and vegetables and for a number of extracts and concentrates made from them, including crystalline hesperetin, the aglycone of hesperidin and various samples of hesperidin chalcone. It was stated there that the considerable variations found for the latter substance could well be due to its instability and impurity, and it was also recorded that a number of synthetic compounds having structures based on the aglycosidic chalcone skeleton had in no instance given unequivocal indications of activity.

The general principles governing the bioassay of vitamin P, simple to state and, we believe, to grasp, have unfortunately not proved so simple to follow. The application of the modified Zacho method (Bacharach *et al.*, 1942) to quantitative bioassay of vitamin P activity has been found difficult or impossible to apply by a number of authors. Workers both at the California Fruit Growers' Exchange and at Abbott Laboratories have not always been able to make the test work (personal communications) and nor have we (unpublished results). There is here perhaps a somewhat ironical analogy with Szent-Györgyi's failure to confirm the vitamin's very existence. Nevertheless, we are satisfied that our original findings of a linear relationship between "critical petechial pressure" and "log dose" of vitamin P is valid over a range adequate for bioassay. We must therefore conclude that any failure of others or ourselves to repeat the test is due to the incursion of unknown and uncontrolled, but not essentially uncontrollable, variables.

In our original publication we emphasized the sensitivity of the guinea pig's capillary resistance to temperature and damage: one of us has called attention to the effect of extravascular suffusion of blood on capillary resistance (Scarborough, 1941) in human subjects, and the effect is doubtless also to be demonstrated in guinea pigs. There may, moreover, be unrecognized dietary variables involved. This is, indeed the view of the French school. On the one hand they report that the production of vitamin P deficiency in guinea pigs is affected by the variety of oats in the basal diet, "avoine grise" being satisfactory; they suggest that the unsuspected presence of vitamin P activity in "avoine blanche" may have prevented some workers from producing the required deficiency in guinea pigs. On the other hand, they claim to have demonstrated the presence in certain vegetable extracts of substances having an antivitamin P action and mention the turnip as a source (Parrot and Cotereau, 1945).

A detailed reinvestigation of the method of biological assay is, we suggest, basic to any real advance in understanding the nature and function of vitamin P or even, according to the more sceptical view, to proving its existence. Such a study would need to examine both the alternative guinea pig method of Bourne (1943) and the use of rats, as advocated by Rusznyák and Benko (1941a), or rabbits (Thienes *et al.*, 1942), as well as our more fully described modification of Zacho's technique. When this has been done, then and only then, assuming that the existence of a substance preventing and curing dietary capillary fragility is confirmed, along with a method for its reasonably accurate measurement, will it be possible to look into the validity of alternative and short-cut methods of assay. These include methods for measuring bleeding time (Ungar, 1944; Parrot and Galmiche, 1945); the procedure involving the effect of low pressure on mice (Majovski *et al.*, 1944), though this would seem to have been given a lasting quietus by Kibrick and Goldfarb (1944) and by Raiman and Necheles (1947); enzymological methods, such as those based on the inhibition of succinoxidase (Bartlett, 1948) and hyaluronidase (Beiler and Martin, 1947); and a number of pharmacological properties (e.g., protection against toxicity of arsenicals (Friend and Ivy, 1948)). In addition, the availability of a sound bioassay technique, reasonably precise and accurate, would make possible some valid assessment of the various chemical tests that have been put forward from time to time, but can so far rest only on the shifting sands of an indeterminate biological foundation.

Alongside this work should go further attempts to evaluate the nature and importance of vitamin P deficiency in man, and this would of necessity involve giving the most careful consideration to the experimental data and arguments put forward by G. H. Bell and his colleagues (Bell *et al.*, 1940; Munro *et al.*, 1947–8; Lazarus *et al.*, 1948). The assay of a physiologically active substance, or group of substances, in foods has direct relevance only to the needs of the species of experimental animal used in the test: application of the knowledge so acquired to another species can only be justified by valid experimental work. True, a substance proved to be needed by divers species is more likely to be needed by another than one that has been tested only on a single kind of laboratory animal, but one cannot know until one has tried. However far we may go in establishing the needs of guinea pigs, and perhaps also of rats and rabbits, for vitamin P, its importance to man can only be established by trials on man. Studies of the techniques for measuring capillary resistance in human beings seem to us to be legitimately considered under the present heading, for without them the figures afforded by assays cannot have any known bearing on human requirements.

2. Chemical Analysis

All the methods so far devised for the chemical determination of vitamin P, whether in vegetables and fruits, and extracts prepared from them, or in animal tissues and excreta, have been founded upon one or other of a number of color reactions. It must be admitted at once that none of these procedures is really satisfactory. Although they may perhaps give an approximate idea of the total concentration of flavonoid—and even this is doubtful—they are certainly not specific for any particular compound, and it is misleading, therefore, to speak of the hesperidin or eriodicytol content of a foodstuff. It is even more clearly a mistake to refer to the "citrin" concentration in an extract, for "citrin," although it has been used as a standard for colorimetric comparisons, is not itself a product of proven constant composition. Least justifiable of all is to interpret the results of those color tests as measuring vitamin P activity.

The first chemical procedure to be used to any extent is that of Lorenz and Arnold (1941), which was originally devised to measure the vitamin P content of lemon extracts. It is quite unsuited to the determination of flavonoids in urine. Lorenz and Arnold based their test on the red color produced by heating "citrin" solutions with potassium hydroxide, the color being compared with an 0.05 *N* solution of iodine itself carefully standardized against "citrin" solutions prepared in two different laboratories. The determination was made on extracts of whole lemons, or of lemon peel, extracted with boiling water for 10 minutes. This method was used fairly extensively by the Chinese workers, with results already summarized. It was subsequently modified by Goldfarb *et al.* (1942) because they found that fermentable reducing monosaccharides, present to about 1% in lemon juice and extracts of lemon peel, produced a measurable color with their KOH solution, especially after boiling. They therefore recommended that extraction be by water at room temperature for 24 hours, rather than by boiling water for 10 minutes as advocated by Lorenz and Arnold, and employed a solution of "eriodictyol glycoside" as colorimetric standard.

In an attempt to study the "flavanone content" (*sic*) of various tissues Huszák (1937b) employed the cyanidin reaction. Tissues were extracted with 3 volumes of 90% alcohol for 20 minutes under reflux, the extract was made faintly acid and concentrated to dryness; the solid residue was then re-extracted with a volume of 90% alcohol equal to the volume of the tissue. To this was added one-third volume of concentrated hydrochloric acid and magnesium shavings. The reacting mixture was kept cool and the resulting violet color compared with that of a "citrin" solution similarly treated. This method has yielded the only figures available for the flavonone content of tissues before and after the administration of "citrin" to animals.

Armentano *et al.* (1938) and Neuweiler (1939) employed the so-called silver lactate method to determine the "citrin" content (*sic*) of human urine. The yellow urinary pigments are removed by the addition of a solution of silver lactate. After centrifugation an aliquot of the supernatant fluid is treated with 5% sodium cyanide and 20% sodium carbonate. The brownish-red color of the solution after 24 hours is compared photometrically with the color of a standard solution of citrin similarly treated. This method seems to have been extensively used by the authors, in whose hands it apparently gives satisfactory results, in spite of the fact that, as they admit, their standard is not a single substance or even a constant mixture of substances. Since eriodictyol glycoside gave a much deeper color than hesperidin, they consider that the method determines chiefly the former. In our hands this procedure has proved of no value. We were unable to remove all traces of yellow from the urine by silver lactate. Furthermore, the color produced by the addition of alkali varied greatly in different specimens of urine and with the time for which it was allowed to stand. Keeping the solution 12–24 hours before comparison usually resulted in the formation of a colored precipitate, and color matching soon after addition of alkali was rendered inaccurate and difficult by a fairly rapid alteration both in depth and in tint. Finally we were rarely fortunate enough to obtain exactly the same color in standard and unknown.

Lindheimer *et al.* (1942) employed these three methods simultaneously to measure the vitamin P content of lemon rind, potatoes, spinach, green peppers and cabbage, using a preparation (noncrystalline) of eriodictyol glycoside as a standard. The three methods gave very variable results and showed poor agreement. Our own experience with the three tests in the determination of vitamin P compounds in urine has not been at all encouraging. It is possible roughly to measure the hesperidin content of solutions by the cyanidin reaction, but this became quite unsatisfactory in our hands when applied to biological fluids.

Perhaps more encouraging are the observations of Wilson, *et al.* (1942) based upon the boric acid reaction described by Wilson (1939), which apparently is specific for flavones. This method has been used by Weatherby and Cheng (1943) to determine quercetin-like substances in certain plants. It has the disadvantage that it requires air-dried material. The dried sample is extracted with methanol and after evaporation and removal of chlorophyll and other pigments by extraction with chloroform the residue is taken into acetone. To this solution is added the boric-citric acid reagent and the resulting yellow color is measured in a photoelectric colorimeter with a blue filter. Reagent blanks are made and the flavone equivalent (*sic*) is determined by reference to a curve previously established with pure quercetin. This method has been applied to human urine by Kirtley and Peck (1948) using water-soluble "hesperidin methyl chalcone" as the standard. They were able to recover 99.6% of the chalcone added to urine.

It is difficult to evaluate the significance of much of this work because the unspecificity of the reactions and the variety of colorimetric standards render it impossible to say what exactly is being measured. The severe limitations of all such procedures must render almost valueless statements made at the present time about the vitamin P content of biological materials as determined by chemical analysis.

VII. DISTRIBUTION OF VITAMIN P

From what has been written above about assay, it must follow that comparatively few trustworthy data are available about the distribution of "vitamin P" in animal and vegetable foods. The figures quoted below, almost entirely from our own publications, are themselves to be taken with reserve. In no instance was a detailed study made—nor could it be—of even a single food: the results, at best on two or three random samples, are not necessarily average or representative in the country (England) where the samples were collected. They may by chance be figures from the extreme ends of the histograms relating concentration to distribution. Nevertheless they are the only figures available and have at least the saving grace that they are all related to a reasonably fixed "unit," however provisional, which was the activity of 1 mg. of a particular water-soluble concentrate made from orange meal. Although subsequent experience has shown (unpublished) that such concentrates are probably unstable (when kept over sulfuric acid in an unevacuated desiccator) and therefore unsuitable even as provisional standard preparations, the concentrate used as the standard has been carefully compared with crystalline hesperidin, of which the purity was expressed in terms of its specific absorption in the ultraviolet (for pure hesperidin, $E^{1\%}_{1\text{cm.}}$ 285 μ = 335; a sample of recrystallized hesperidin, having 93% of this activity, was found to be equivalent to 95 units per g., with limits of activity, at the 5% level, of 70 to 130). Here, at any rate is a reference point, even if a somewhat indirect one, for subsequent determinations of vitamin P potency.

Before tabulating available data for the vitamin P activities of fruits and vegetables, it is necessary to say something about the claims made for the activity of a number of pure, or believedly pure, compounds. We have already referred above to the claims for methylated hesperidin chalcone, for rutin and for *d*-epicatechin. In our experience the first two of these have, respectively, appreciably greater and slightly less activity than hesperidin itself, but the experimental error of the guinea pig test is sufficiently large for these substances, as tested by us, to have differed little in their effects on capillary resistance. The statement applies *a fortiori* to tests on human subjects and to epicatechin for, to the best of our knowledge, no statistically evaluated comparison of this with hesperidin (by the oral route) has been recorded adequately (if at all) in the literature. What may, perhaps, be legitimately repeated here is that no account of the occurrence and nature of vitamin P can be held satisfactory unless it provides for the existence in black currants of an extremely potent compound, or group of compounds, having more than one hundred times the activity of recrystallized hesperidin—always assuming that the published results to that effect can be confirmed!

All the figures in Table I apply, perforce, to British grown plants: most of them are taken from the paper by Bacharach and Coates (1943). The figures are, in several instances, the merest approximations, for reasons given in the original publication, but we nevertheless believe that the overall picture is not too far removed from the truth. The results of an independent assay (Scarborough, 1945) of certain foodstuffs on human subjects are given in the last column for comparison. We regard the figure given for whole black currants as anomalous, for reasons stated already (Bacharach and Coates, 1944).

After reiterating two comments made in the original paper, that there is no apparent correlation between the contents of ascorbic acid and vitamin P activity or between the latter and anthocyanin or other plant pigments, and that on the whole fruits are more active than leaf or root vegetables, we must add that our finding of appreciable, if small, activity in the turnip is superficially at variance with the statement of Parrot and Cotereau (1945), noted above. It may also be repeated that the richest concentrate so far described is that from black currants (Pollard, 1942) (for we hold that the French workers' evaluation of *d*-epicatechin affords us no basis for comparison with substances assayed by the oral route) and that several rich concentrates have also been made from rose hips (Wokes *et al.*, 1943).

VIII. CONCLUSION

Although there are still a number of biological effects attributed to vitamin P that we have not mentioned, we have probably said enough to make it plain that there are many confusing and contradictory aspects of the present position. Clearly the main purpose of further study should be to establish some unified theory of the mechanism of action of these compounds which will serve to explain and link together their effects in increasing capillary resistance, in potentiating the action of adrenaline and perhaps also in reducing histamine sensitivity. The clue to this problem may possibly be found in the answer to the question: do flavonoids exert any direct action on the capillary vessels and, if so, what is the nature of this action? It is probable that clinical investigation has little to contribute to such a study, although it might provide indirect evidence that the apparent increase in capillary resistance which follows the administration of, for example, hesperidin is the result of real alteration in the resistance of capillary walls to stress, or that it is due to changes in the pattern of blood flow through capillary beds. In the matter of capillary resistance this is the crucial question on which more information is required. Because, on the whole, the determination of capillary resistance is more satisfactory in man than in laboratory animals, further clinical studies directed towards determining the relationship between the changes in capillary resistance, apparently produced, for example, by heat and cold and by vasodilator and vasoconstrictor drugs, might be of some value, although it is clear that the evidence furnished by such studies can be no more than indirect. They will clearly require the use of a method which is capable of giving fairly frequent determinations of capillary resistance and the most can be used under stringent experimental conditions. We believe that only with a negative pressure method can carefully standardized conditions be obtained. For animal studies the rat meso-appendix preparation appears likely to afford a valuable method of study, although here again it will be necessary to control experimental conditions with care. In particular it would seem desirable to explore the possibilities of using this approach in animals from which sources of endogenous adrenaline production have been removed and, in view of the large doses of flavonoid which may be used, to record the arterial pressure continuously. Frequent determinations of the temperature of the fluid bathing the preparation will also be required. Finally, it still remains to be shown that such biological effects as have already been attributed to this group of compounds are those of a vitamin. Much of the more recent work has employed doses whose size suggests a pharmacological action rather than that of a vitamin. On the other hand, the laboratory studies of Zacho, of Bourne, of Hughes and Parkes and of Bacharach, as well as the clinical studies of Scarborough, seem to establish that the action on capillary resistance is that of a vitamin. Whether an unduly low capillary resistance confers any disadvantage on the organism is another question which also remains to be decided. For these reasons the results of a study directed towards producing a deficiency syndrome in the guinea pig will be of much significance.

One last point requires emphasis. Any further studies to be made either in the laboratory or in the clinic require the use of pure, preferably synthetic, compounds whose constitution has been determined. Too frequently in the past has the interpretation of results been prejudiced by the use of preparations that are not homogeneous and whose constitution is at best approximately known.

TABLE I

The Vitamin P Activities of Some Plants and Plant Products

A "Provisional Unit" (P.U.) is defined as the biological activity, on the vitamin P deficient guinea pig, of 1 mg. of a citrus concentrate (WSP1), the laboratory Provisional Standard Preparation. Recrystallized hesperidin showing the maximum ultraviolet absorption recorded had almost exactly 100 P.U. per g..

The figures given in the table are for the most part taken, with some minor additions and modifications, from Bacharach and Coates (1942, 1943, 1944) and Scarborough (1945).

A. FRUITS AND VEGETABLES

Species	Material	Portion	Provisional units per g.	
	Description		Guinea pig test	Clinical assay
1. Apple (*Pyrus malus*)	Bramley's seedling	Fruit	0.6	
2. Apricot (*Prunus armeniaca*)	Dried	Fruit	....	0.75 to 1.0
3. Beetroot (*Beta vulgaris*)		Root	0.15	"Negligible"
4. Bilberry (*Vaccinium mystillus*)		Fruit	....	1.0
5. Blackberry (*Rubus fruticosus*)		Fruit	0.6	0.8 to 1.0
6. Black currant (*Ribes nigrum*)	Purée (calc. to original fruit)	Fruit	5.0 to 7.3	3.0 to 4.5
		Fruit	(0.75?)	
7. Cabbage (*Brassica oleracea*)	var. capitata April	Leaf	0.6	< 0.5
	var. capitata October	Leaf	1.0	
8. Carrot (*Daucus carota*)	April	Root	0.1	"Negligible"
	August	Root	0.4	
9. Cauliflower (*Brassica oleracea*)	var. *botrytis cauliflora*	Flower	0.4	< 0.5
10. Cherry (*Prunus cerasus*)	Black	Fruit	0.6	1.0
	White	Fruit	0.5	
11. Dandelion (*Leontodon taraxacum*)		Leaf	0.3	
12. Dock (*Rumex acetosa*)		Leaf	0.2	
13. Grape (*Vitis vinifera*)	Black	Fruit	....	5 to 10
	White	Fruit	....	5 to 10
14. Grape-fruit (*Citrus grandis*)		Fruit	....	1

15. Lemon (*Citrus limonum*)		Fruit		5
	Italian[1]	Juice	4.5	5 to 7.5
	Italian	Peel	5.0	
16. Lentil (*Ervum lens*)		Seed	0	
17. Lettuce (*Lactuca sativa*)	Cabbage[1] type, May[1]	Leaf	0.8	< 0.5
	Cabbage[1] type, September[1]	Leaf	1.0	
18. Maple pea (*Pisum arvense*)	Dried	Seed	0.1	
	Germinated *ex* dried	Seed, etc.	0.1	
19. Orange (*Citrus aurantium*)	South African[1]	Fruit		3.0 to 5.0
	South African[1]	Juice	4.7; 5.1	3.0 to 6.0
		Peel	4.4; 5.5	2.0 to 3.0
20. Parsley (*Petroselinum sativum*)		Leaf	1.3	
21. Parsnip (*Pastinaca sativa*)		Root	0.4	"Negligible"
22. Pea (*Pisum sativum*)	Harrison's Glory—dried	Seed	0.4	
	Harrison's Glory—germinated *ex* dried	Seed, etc.	0.8	
23. Plum (*Prunus domestica*)	Victoria	Fruit	0.5	0.5 to 1.0
	Blue	Fruit		1.0 to 2.0
24. Potato (*Solanum tuberosum*)	Old[1] April[1]	Tuber	0.25	"Negligible"
	Old[1] July[1]	Tuber	0.40	
25. Prune	Dried	Fruit		3.0 to 4.0
26. Rose (*Rosa arvensis*)		Fruit—flesh	6.8	
(*Rosa coriifolia*)		Fruit—flesh	5.6	
(*Rosa canina*)	Essex	Fruit—flesh	2.4	
27. Rowan (*Sorbus aucuparia*)	var. *asplenifolia*	Fruit	3.0	
28. Rhubarb (*Rheum rhaponticum*)		Stem	0.2	
29. Spinach (*Spinacia oleoracea*)	May	Leaf	1.3	
	September	Leaf	1.3	
30. Swede (*Brassica campestris*)		Root	0.2	
31. Tomato (*Lycopersicum esculentum*)	July[1]	Fruit	0.7	0.5
	August[1]	Fruit	0.6	

TABLE I.—(*Continued*)

A. FRUITS AND VEGETABLES

Species	Material Description	Portion	Provisional units per g. Guinea pig test	Provisional units per g. Clinical assay
32. Turnip (*Brassica rapa*)	June[1]	Root	0.2	"Negligible"
	July[1]	Root	0.3	
33. Walnut (*Juglans niger*)	Black	Kernel	1.0	
34. Watercress (*Nasturtium officinale*)	April	Shoot	0.1	
	October	Shoot	0.7	

B. EXTRACTS, CONCENTRATES, ETC.

Description	Guinea pig test	Clinical assay
Citrus concentrate (WSP1)—by definition	1000	1000
Citrus concentrate (WSP2)	1680 (F.L. 1120 to 2490)[2]	
Rose hip syrup	3.5	1.0 to 6.0 (5 Samples)
Black-currant puree	4.8	2–6
Black-currant concentrate PB	47	
W	52:38 (Duplicate assay)	
WSP4	10,600 (F.L. 8375 to 13,360)[2]	3000
"Citrin" (from whole lemon by Szent-Györgyi's method)		650
Hesperidin (93%, by ultraviolet absorption, m.p. 265–266°C.)	95 (F.L. 70 to 131)[2]	100
Hesperetin (m.p. 223–224°C.)	75 (Very rough assay)	25–50
Hesperidin chalcone (or its Na salt)	400 to 2700	
Methylated hesperidin chalcone	Approx. = hesperidin chalcone	
Rutin	Approx. = hesperidin	50

[1] Applies to sample for guinea pig test only.
[2] F. L. = fiducial limits at P, 0.05.

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Stereoisomeric Provitamins A

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I. INTRODUCTION

An important problem in the field of the vitamins is to establish precise and quantitative relationships between chemical structure and bio-activity. However, such studies which are restricted to the use of two-dimensional structural chemistry cannot cover this whole field in the class of the provitamins A; one also wishes to learn to what extent their bio-potency is affected by steric variations in the molecules.

As is well known, the provitamins A belong to the carotene-like polyene pigments, the so-called "carotenoids." It was recognized early by Steenbock *et al.* (1921) that "carotene of constant melting point through a number of crystallizations was always found to induce growth in rats"; and it was subsequently shown by Euler *et al.* (1928) that the surprisingly small daily carotene dose of 5 γ was effective in this animal. For further developments and discussions we may refer to the survey presented by Heilbron *et al.* (1944).

All carotenoids contain extended conjugated double bond systems in an open carbon chain. Such systems are not only subject to *cis-trans* isomerization but they also constitute a unique case in stereochemistry because of the unusually high number of the possible stereoisomers. Let us consider the four provitamins A which are practically most important and to which the present survey is restricted, viz., the three $C_{40}H_{56}$-hydrocarbons, α-, β-, and γ-carotene, and a monohydroxy-β-carotene, termed cryptoxanthin, $C_{40}H_{55}\cdot OH$. When we pass from a two-dimensional to a three-dimensional treatment, the probable number of these provitamins increases from 4 to 148. Only relatively few of the possible spatial forms are known at the present time, and still fewer have been assayed on animals. However, the available experimental data prove clearly that any spatial alteration does change the bio-potency of a provitamin A without destroying it.

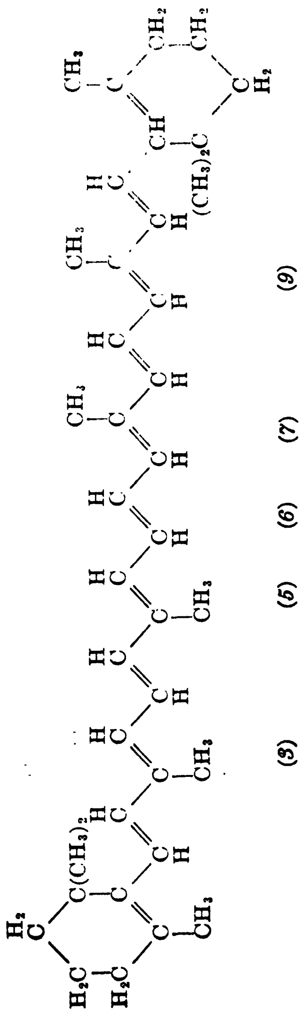
Generally speaking, in current biological studies of stereoisomeric compounds either optical isomers (having different configurations around asymmetric carbon atoms) or else *cis-trans* isomers are being considered. From the viewpoint of molecular morphology there is a great difference between these two types of stereoisomerism. In the epimerization of a sugar, for example, when a hydrogen and a hydroxyl that are attached to the same carbon atom interchange their positions, only a detail of the molecule, not its over-all shape, is markedly altered. In contrast, when a centrally located double bond, in a long, aliphatic, conjugated, all-*trans* double bond system, assumes *cis*-configuration, the whole molecular pattern is fundamentally altered: instead of the initial, evenly zig-zagging main carbon chain and, as a whole, straight shape of the molecule, the latter assumes a V-like form.

It is understandable that such deep-seated morphological changes do influence the *in vivo* behavior of the compound since each spatial form of the molecule may not fit equally well into a given biological system in which the compound is found to be active. This postulate is in accordance with the *in vitro* observation that any *cis-trans* rearrangement of a carotenoid causes substantial changes in its fundamental physical characteristics such as adsorption affinity and spectrum.

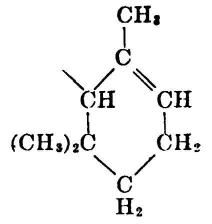
II. SOME PHYSICAL-CHEMICAL FOUNDATIONS

At this point it is necessary to summarize some aspects and methods which have been used in the study of stereoisomeric provitamins (and other carotenoids) previous to the bioassays which will be considered in a subsequent section.

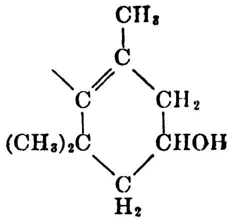
According to the formula, the β-carotene molecule contains 11 conjugated double bonds. The two terminal double bonds are not subject to spatial changes since they are located in rings. As a rule, the other double bonds are all in *trans* configuration in naturally occurring β-carotene. Of these, as pointed out by Pauling (1939), four double bonds are sterically hindered from rotating into *cis* position, namely, those of which one carbon atom is adjacent to a C—CH_3 group, since the relatively voluminous methyl involves a spatial conflict with a hydrogen atom. Consequently, of the 11 double bonds only five are "sterically effective," viz., those numbered in the β-carotene formula.



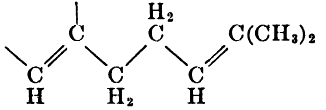
All-*trans*-β-carotene
(Only the double bonds (3), (5), (6), (7), and (9) are sterically unhindered.)
(In reality, the C═C—C angle is 125° 16′.)



α-Carotene
(one terminal group)



Cryptoxanthin
(one terminal group)



γ-Carotene
(one terminal group)

This restriction as well as the symmetrical structure of β-carotene allows only the 20 *cis-trans* isomers whose carbon skeletons are depicted in Fig. 1. α-Carotene, γ-carotene, and cryptoxanthin may assume considerably more spatial forms as indicated in Table I. Further details may be obtained from two reviews (Zechmeister, 1944, 1947).

TABLE I
Stereoisomeric Provitamins A

Compound	Number of spatially effective double bonds	Symmetry of molecule	Probable number of *cis-trans* isomers
α-Carotene	5	Non-sym.	32
β-Carotene	5	Sym.	20
γ-Carotene	6	Non-sym.	64
Cryptoxanthin	5	Non-sym.	32

The totality of all stereoisomers of a given compound is termed a "stereochemical set" and each stereoisomer is a "member" of the set.

Most fresh plant extracts contain each of the provitamin A molecules in all-*trans* configuration. The latter is, however, protected from stereochemical rearrangements only in the tissue or in a crystal grating. In solution (or in the molten state) the morphological uniformity of the carotenoid molecules is soon broken and a mixture of stereoisomers is formed in which the unchanged portion of the all-*trans* compound mostly predominates; for example, two-thirds of the molecules may retain the all-*trans* configuration while one-third is present as a newly formed mixture of *cis* compounds.

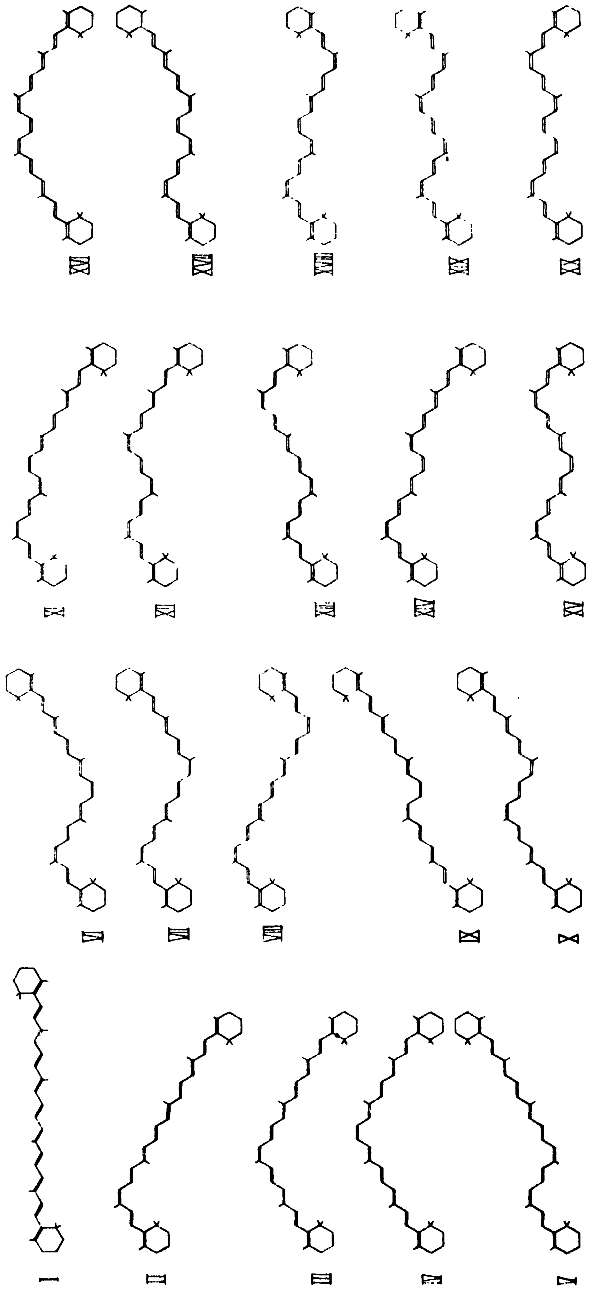


FIG. 1. Skeleton models of the twenty unhindered stereoisomeric β-carotenes. I—all-*trans*-β-carotene; II-IV—mono-*cis* β-carotenes; V-X—di-*cis* β-carotenes; XI-XVI—tri-*cis* β-carotenes; XVII-XIX—tetra-*cis* β-carotenes; XX—penta-*cis* β-carotene.

Such a bending of the initially straight molecules progresses spontaneously but slowly when a solution is kept at room temperature, much more rapidly upon refluxing, and, especially conveniently for the experimenter, during a few minutes' illumination in the presence of catalytic amounts of iodine (Zechmeister and Tuzson, 1938, 1939). The photochemical isomerization of solutions in the absence of catalysts, for example, by exposure to sunshine etc., is likewise effective.

These manifold phenomena alone would scarcely have allowed rapid progress in the isolation and biochemical study of the individual provitamin A stereoisomers, since complex mixtures are formed in each case. However, the sharp dependence of the adsorption affinity on the shape of the molecule made the resolution of these mixtures possible. While an ordinary, all-*trans* carotenoid forms a single zone on the chromatographic column, several colored zones appear after any of the isomerizing treatments mentioned have been applied. Indeed, it was possible to observe 13 to 14 stereoisomeric β-carotene zones (Polgár and Zechmeister, 1942). The stereochemical purity of each zone, i.e., the absence of other stereoisomers, can be attained and tested by re-chromatography on a fresh column; and in some cases the spatial configuration can be preserved by crystallization. In solution, at 0°, in the absence of air and light, even relatively labile configurations may be preserved for a long time. Furthermore, since each member of a given stereoisomeric set yields the same equilibrium mixture upon iodine catalysis, a quantitative estimation of noncrystallizable stereoisomers is also possible within reasonable limits of error. This is made on the basis of extinction data which were obtained by catalyzing a weighed sample of any crystalline member of the set.

The determination of the individual configurations is a difficult task. However, there are some optical methods available by means of which the solution of this problem becomes possible, at least with a high degree of probability. It was noticed first by Gillam and El Ridi (1936) that an isomerization of ordinary β-carotene caused a weakening of the color intensity. Many similar observations were then made by our group, and a theoretical interpretation of this phenomenon was given by Pauling (1939). Within each stereochemical set the all-*trans* form possesses the strongest chromophore; and if it undergoes a stepwise *trans* → *cis* rearrangement, the wave length positions of its spectral maxima are shifted gradually towards the ultraviolet region (Zechmeister, LeRosen, Schroeder, Polgár, and Pauling, 1943). From the extent of this shift one can estimate approximately the number of the *cis* double bonds present in a given steric form (cf. Zechmeister, 1944). These conclusions have been confirmed by the isolation and study of some naturally occurring carotenoids such as pro-γ-carotene (a provitamin A), and several members of the biologically inactive lycopene set which contain four to seven *cis* double bonds (LeRosen and Zechmeister, 1942; Zechmeister and Pinckard, 1947).

Another spectral phenomenon which allows an insight into the shape of the molecule is the "*cis*-peak effect" (Zechmeister and Polgár, 1942). As Fig. 2 shows, the extinction curve of all-*trans*-β-carotene undergoes, upon refluxing or iodine catalysis (in light), two characteristic changes, besides the wavelength shift just mentioned. In the visible region the height of the maxima and the degree of the fine structure decrease but at the same time a new maximum grows out in the invisible region, around 340 mμ. Subsequent chromatographic resolution showed that mainly one stereoisomer is responsible for the appearance of this maximum (Zechmeister and Polgár, 1944).

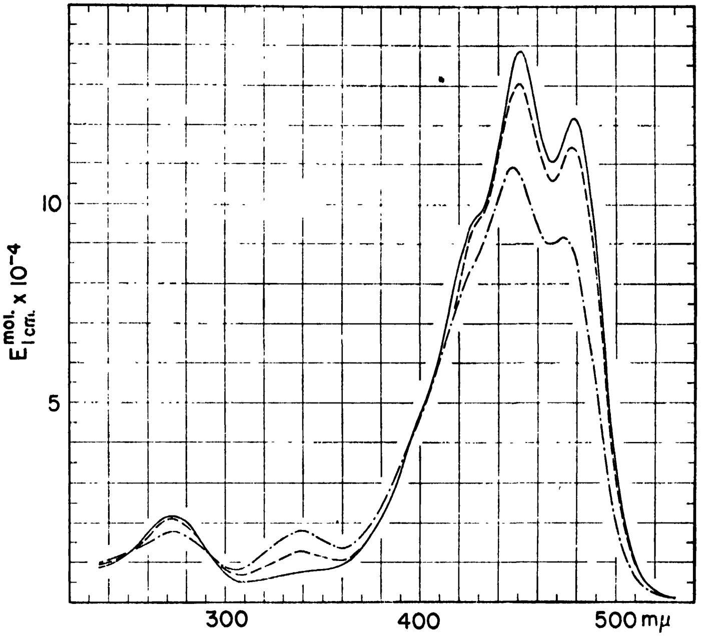


FIG. 2. Molecular extinction curves of β-carotene in hexane:——, fresh solution of the all-*trans* compound,- - -, mixture of stereoisomers after refluxing in darkness for 45 minutes; -·-·, mixture of stereoisomers after iodine catalysis at room temperature, in light. (From *J. Am. Chem. Soc.* **65**, 1523, 1943.)

The *cis*-peak effect is a phenomenon which has been interpreted as the result of the oscillation of the electrons from the two ends of the conjugated system toward the middle and vice versa. In a V-shaped molecule this gives rise to a dipole moment which is perpendicular to the straight line connecting the ends of the conjugated system. In a rod-like molecule (e.g., all-*trans*-β-carotene) no such effect takes place and the spectral curve is flat in the *cis*-peak region. As a rough approximation, the height of the *cis*-peak is proportional in each case to the square of the distance between the center of the chromophore and the mid-point of the straight line between its ends (Zechmeister, LeRosen, Schroeder, Polgár, and Pauling, 1943).

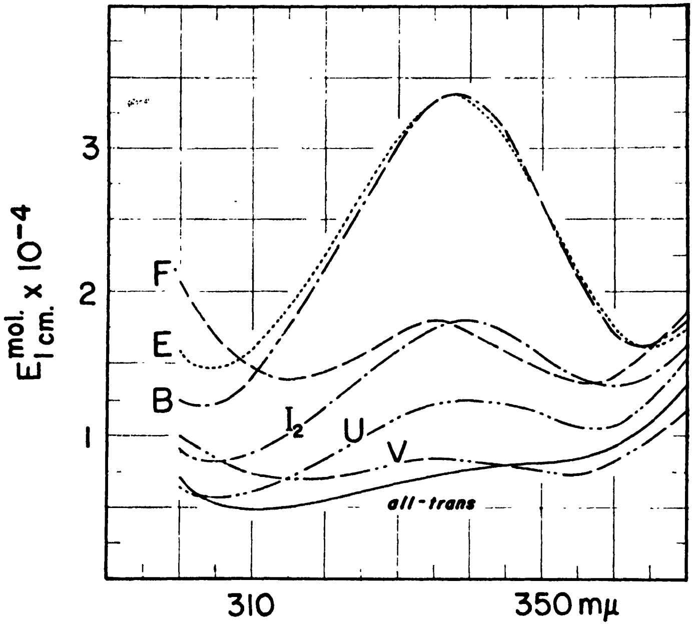


FIG. 3. Molecular extinction curves of some members of the stereoisomeric β-carotene set in the *cis*-peak region, in hexane. I_2 indicates the equilibrium mixture formed upon iodine catalysis, at room temperature, in light. (From *J. Am. Chem. Soc.* **66**, 139, 1944.)

From such data the general form of a stereoisomer can be deduced since in principle each individual stereoisomer shows a *cis*-peak which is characteristic for its configuration (Fig. 3). According to Fig. 1, only a few members of the stereoisomeric β-carotene set can show a high *cis*-peak. For example, in a molecule corresponding to X its appearance is prevented by the compensation of two opposite dipoles; neither are the requisites present for the formation of a *cis*-peak in poly-*cis* compounds like XIX or XX since the presence of many *cis* double bonds straightens the molecule out.

On the basis of the concepts and observations just outlined, the following stereochemical types of provitamins A became available for biological research.

(*a*) All-*trans* compounds: from natural sources, well crystallized, having maximum color intensity, maximum fine structure of the spectral curve in the visible region but no *cis*-peak;

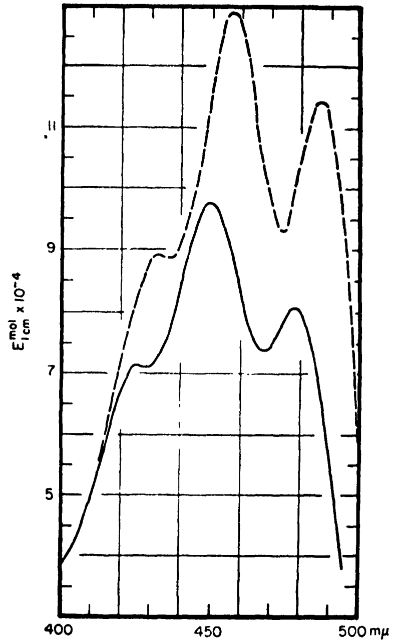


FIG. 4.

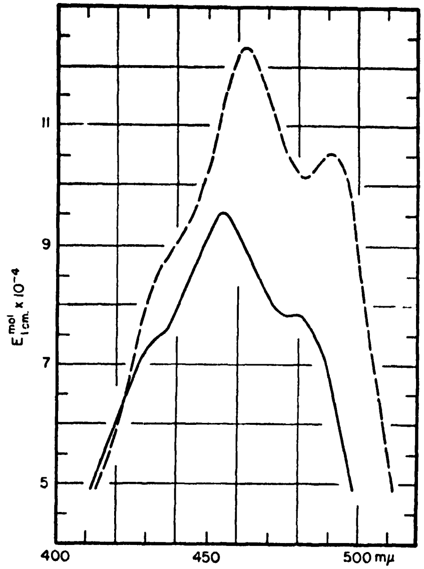


FIG. 5.

FIG. 4. Molecular extinction curves in Wesson oil: —, neo-α-carotene B; and ---, all-*trans*-α-carotene. (From *Arch. Biochem.* **7**, 247, 1945.)

FIG. 5. Molecular extinction curves in Wesson oil: —, neo-β-carotene B; and ---, all-*trans*-β-carotene. (From *Arch. Biochem.* **7**, 247, 1945.)

(*b*) peripheral mono-*cis* forms: artifacts; well crystallized, showing moderate *cis*-peaks; adsorbed above the corresponding all-*trans* compound on the Tswett column;

(*c*) mono- and di-*cis* forms with a centrally located *cis* double bond: artifacts; showing tendency to rearrange during crystallization; high *cis*-peaks; adsorbed below the corresponding all-*trans* compound; and

(*d*) poly-*cis* forms: obtainable from natural sources only at present; well crystallized; much weaker in color and having much less fine structure in their spectral curves than either of the foregoing three types, and no *cis*-peak; adsorbed much below the types (*a*)–(*c*).

III. METHODS OF BIOASSAY

In this section mainly those procedures will be considered which were used recently under the guidance of Professor H. J. Deuel, Jr. of the University of Southern California and the present writer, by our colleagues, C. E. Calbert, A. Chatterjee, T. Fukui, S. M. Greenberg, C. Hendrick (Mrs. Johnston), E. R. Meserve, J. H. Pinckard, A. Polgár, A. Sandoval, W. A. Schroeder, E. Straub, and E. Sumner (Deuel *et al.* 1944a, b; 1945a, b, c; 1946; 1947).

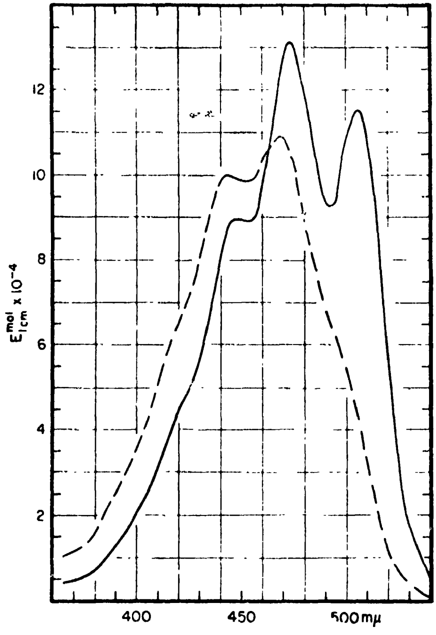


FIG. 6.

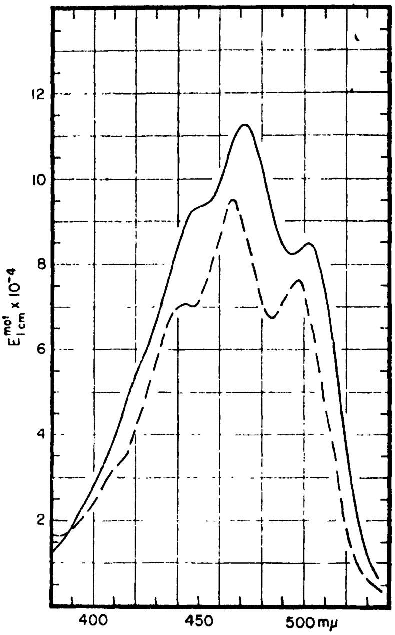


FIG. 7.

FIG. 6. Molecular extinction curves in Wesson oil: —, all-*trans*-γ-carotene; and - - -, pro-γ-carotene. (From *Arch. Biochem.* **14**, 97, 1947.)

FIG. 7. Molecular extinction curves in Wesson oil. —, neo-γ-carotene P; and - - - mixture of some stereoisomers adsorbed below the all-*trans* form. (From *Arch. Biochem.* **14**, 97, 1947.)

Compared with routine estimations of vitamin A potency, an additional complication appeared in these experiments, viz., the requirement that the initial stereochemical configuration of the supplements must be preserved and guaranteed throughout the assay period. Spatial rearrangements had to be prevented. This was not difficult considering the relative constancy of the tested *cis* configurations in Wesson oil, in the presence of α-tocopherol. When an undesirable spatial rearrangement does occur, it is directed toward the creation of a quasi-equilibrium mixture of stereoisomers in which the newly formed all-*trans* compound gradually becomes preponderant.

Fortunately, there is a sufficient spectral difference in Wesson oil solutions between any *cis* form assayed and the corresponding all-*trans* isomer to permit a ready check of stereochemical homogeneity. As demonstrated in Figs. 4–7, even a 5% isomerization would be noticeable in the Beckman instrument by the increase of the maximum extinction and its slight shift towards longer wavelengths. As soon as minor changes in this direction were noticed, the solution was replaced by a fresh one. Thus, for 95% (and usually more) of the ingested provitamin A molecules the initial configuration was maintained.

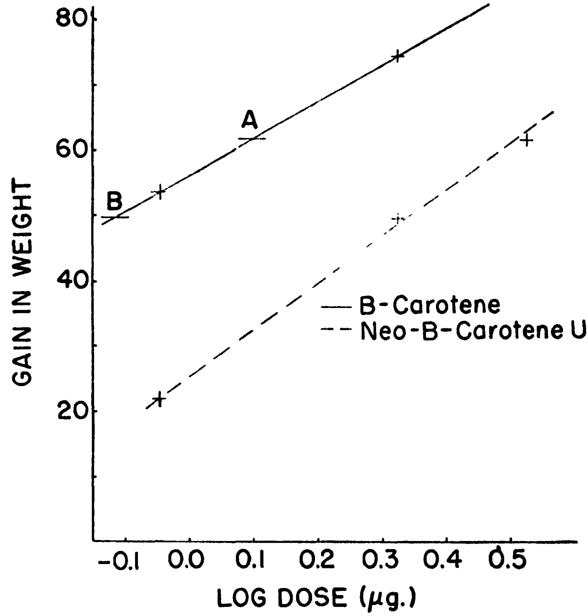


Fig. 8. Relationship of gain in weight to dosage of all-*trans*-β-carotene and neo-β-carotene U. (From *Arch. Biochem.* **5**, 107, 1944.)

All bioassays were carried out in the presence of a constant and relatively high amount of α-tocopherol (0.5 mg./0.1 ml. of oil) which, according to Hickman *et al.* (1942, 1944; cf. Hickman, 1943) allows optimum utilization of carotene (*cf.* Harris *et al.*, 1944).

Since the absolute level of the all-*trans*-β-carotene potency markedly depends on the breed of the white rats (and, possibly, on other factors), a group of animals receiving all-*trans*-β-carotene on at least two levels was run parallel with each assay, beside the usual negative controls. In all about 1500 rats were used.

The relative potency of each compound tested, based on that of all-*trans*-β-carotene, was then calculated by the method of Coward (1938). For example (Fig. 8), the projection of the average gain in weight of the group receiving as daily dose 3.3 γ of neo-β-carotene U, on the β-carotene curve (point A) is located at a point corresponding to a log-dose of 0.110, which is equivalent to a dose of 1.29 γ of all-*trans*-β-carotene. From the ratio of these two doses the relative bio-potencies follow, viz., all-*trans*-β-carotene: neo-β-carotene U = 100:38. As was expected, the three points on the neo-β-carotene U curve fall in a straight line which is reasonably parallel to the all-*trans*-β-carotene curve.

IV. Results of Bioassays

The bioassays were carried out in each of the four stereoisomeric provitamin A sets with about three members, one of which was the all-*trans* compound. Since in the rat all-*trans*-β-carotene is the strongest provitamin A, its biopotency is conveniently accepted as "100% activity" while for each of the other compounds a smaller percentage is characteristic. For some discussions it may be convenient to express the bio-effect of a *cis* provitamin as the percentage of that of its own all-*trans* form.

We shall now survey the quantitative data that are available at the present time in each of the four stereoisomeric provitamin A sets.

1. α-Carotene Set

The relative potencies in rats of all-*trans*-α-carotene and all-*trans*-β-carotene were determined earlier in an important study by Kuhn *et al.* (1933), in order to confirm the then new postulate that *in vivo* a β-carotene molecule gives rise to two molecules of vitamin A while the formation of only one molecule can be expected from α- or γ-carotene, on the basis of their chemical structure. The authors mentioned found that the limiting daily dose termed "Grenzdosis" of α-carotene (or γ-carotene) (5 γ) for the rat was double the corresponding β-carotene dose (2.5 γ). This "Grenzdosis" had been defined as the necessary daily supplement which enabled at least 70% of the depleted animals to increase their weight by at least 10 g. within 35 days. It was not claimed to be a means for an accurate quantitative estimation of the relative bio-potencies of the three carotenes. Indeed, the administration of the "Grenzdosis" of β-carotene produced the desired growth response not in 70% but in 100% of the rats: hence, it may exceed the ideal "Grenzdosis" (which would be required for a 70% response) by an unknown amount.

Euler *et al.* (1934) obtained a different ratio for β-carotene potency: α-carotene potency by the following method of evaluation. Upon feeding 0.75 γ daily, the average increase in weight of the animals receiving all-*trans*-β-carotene was 0.72 g. while the corresponding figure for the all-*trans*-α-carotene containing diet was 0.48 g. On this basis the ratio mentioned would be 3:2.

Considering these discrepancies in the relative potencies and also in the absolute level of the effective doses (the latter perhaps caused by variations of the α-tocopherol content in the food), a re-determination was carried out recently, with the result that all-*trans*-α-carotene shows 53% of the provitamin A potency of all-*trans*-β-carotene (Deuel *et al.*, 1945c).

The first data referring to *cis* members of the stereochemical α-carotene set were given by Gillam *et al.* (1937) who obtained from all-*trans*-α-carotene a "neo-carotene" solution which, upon crystallization, yielded "neo-α-carotene." Neither of these two samples has been subsequently identified with a pure stereoisomer of α-carotene. These authors found neo-carotene to be qualitatively active (four rats) while their neo-α-carotene sample showed about 70% of the bio-potency of all-*trans*-β-carotene (eight rats).

Our group (Deuel *et al.*, 1945a, c) determined the potencies of neo-α-carotene U and neo-α-carotene B. Neo U is a mono-*cis*-α-carotene containing the *cis* double bond in a peripheral location; its best representation is 9-mono-*cis*-α-carotene (or 3-mono-*cis*-α-carotene), the carbon skeleton corresponding to II in Fig. 1. This stable member of the α-carotene set is characterized by the features mentioned above for type (b). In contrast, neo-α-carotene B, another main product of the *in vitro* isomerization, has a more labile configuration and shows the features given above for type (c). It must possess one centrally and one peripherically located *cis* double bond, probably as in VI or VII (Fig. 1). Neo-α-carotene B may have been the main component of Gillam's neo-carotene.

Our assays gave the following results: Neo-α-carotene U shows 13% and neo-α-carotene B shows 16% of the all-*trans*-β-carotene potency.

2. β-Carotene Set

Two *cis* members of this set were tested recently (Deuel *et al.*, 1944a, 1945a), viz., crystalline neo-β-carotene U (Polgár and Zechmeister, 1942), which corresponds in its nature to neo-α-carotene U and is a peripherically mono-*cis* compound (type (c); probably II, Fig. 1); furthermore, a di-*cis* isomer, neo-β-carotene B, another main product of the *in vitro* isomerization and analogous to the neo-α-carotene B (type (c); probably VI or VII in Fig. 1).

In these assays the following provitamin A potencies were found: 38% of the all-*trans*-β-carotene activity for neo-β-carotene U, and 53% for neo-β-carotene B.

Kemmerer and Fraps (1943, 1944) identified our neo-β-carotene U (Polgár and Zechmeister, 1942) with their "carotenoid X," as isolated, for example, from alfalfa extracts. First, they found negative results with reference to a provitamin A activity of their sample (cf. Kemmerer *et al.*, 1944; Kemmerer and Fraps, 1945b); however, later a potency corresponding to 22% and 25% of that of all-*trans*-β-carotene was reported (Kemmerer and Fraps, 1945a).

Neo-β-carotene B was doubtless the main constituent of the historically important, crystalline "pseudo-α-carotene" obtained from β-carotene by Gillam and El Ridi (1936). It is very difficult to prepare chromatographically homogeneous crystals of this compound; however, homogeneous solutions may be obtained easily. Gillam and El Ridi reported that pseudo-α-carotene had a provitamin A potency "of the same order" as all-*trans*-β-carotene (eight rats). According to Beadle and Zscheile (1942), it shows "significantly less" activity than β-carotene; and according to Kemmerer and Fraps (1943) it has "one half the potency of β-carotene." While all these statements are correct and differ only in the intended degree of accuracy, Mann's (1944) remark that "the presence of pseudo-α-carotene in β-carotene solutions does not lessen the biological activity of the solution" should be restricted to relatively small amounts of this *cis* isomer.

3. γ-Carotene Set

As stated above, the bio-potency of all-*trans*-γ-carotene was found by Kuhn *et al.* (1933), using the "Grenzdosis" method, to be one half that of all-*trans*-β-carotene, in accordance with the *in vivo* formation of only one molecule of vitamin A from γ-carotene. Considering the nature of "Grenzdosis" assays, Kuhn's data point rather to a lower efficiency of all-*trans*-γ-carotene than 50% of that of all-*trans*-β-carotene; in fact, in recent experiments we found 28% for a sample *ex Mimulus* (Deuel *et al.*, 1944b).

However, in this stereochemical set, the following reservation should be made.

While all pure samples of β-carotene (or α-carotene) possess identical melting points, analytically and chromatographically pure γ-carotene preparations, originating from different sources, show considerable and unexplained variations in their melting points which extend over the whole range, from 131° to 178° (cf. Zechmeister and Schroeder, 1942a). If the cause of this discrepancy was of crystallographic nature, then, of course, it would not influence the evaluation of the bio-potencies. Since, however, minor structural differences cannot be excluded either for each case, our assays just mentioned in which γ-carotene from *Mimulus longiflorus* Grant (petals) was used, were repeated with a sample originating from *Pyracantha angustifolia* Schneid. (fruit). The result was 26%, in good accordance with that of the first series (Deuel *et al.*, 1947) and with an unpublished value (27%) referring to γ-carotene *ex* tomato paste (Zechmeister, Pinckard, Greenberg, Straub, Fukui, and Deuel).

There occurs in the *Pyracantha* berries a stereoisomer of γ-carotene which, as yet, has not been observed in any artificial isomerization mixture and which was termed neo-γ-carotene P by Zechmeister and Pinckard (1947). Considering its crystallization tendency, stability, and low *cis*-peak, this natural product seems to belong to the type of peripheral mono-*cis* isomers, comparable to neo-α-carotene U and neo-β-carotene U, although it is adsorbed below all-*trans*-γ-carotene on the Tswett column. The provitamin A potency of neo-γ-carotene P was found to be 19% of that of all-*trans*-β-carotene (Deuel *et al.*, 1947).

It was not possible to obtain a pure analog of neo-β-carotene B in the γ-carotene set, i.e., the member with a very high *cis*-peak. Since this set includes many more members than the two other carotene sets, upon isomerization of the all-*trans* compound and subsequent chromatography, a whole group of similar stereoisomers appear on the Tswett column, below the unchanged portion of all-*trans*-γ-carotene (Zechmeister and Polgár, 1945). Because of the similar adsorption affinities, a clear resolution of this group was not successful. Hence, the whole group was tested in the rat and found to possess 16% of the all-*trans*-β-carotene activity (Deuel *et al.*, 1947).

The most interesting stereoisomer of γ-carotene is the naturally occurring poly-*cis* (probably penta-*cis*) compound, pro-γ-carotene, which was isolated by Zechmeister and Schroeder (1942b, c) from some palm fruits and the ripe berries of *Pyracantha angustifolia.* It occurs also in the seeds of *Evonymus fortunei* L. (Zechmeister and Escue, 1942), as well as in some petals (Schroeder, 1942).

Pro-γ-carotene was found to be a powerful provitamin A whose activity amounts to 44% of that of all-*trans*-β-carotene (Deuel *et al.*, 1944b). This would be 1.6 times the potency of all-*trans*-γ-carotene *ex Pyracantha* (26–28%).

Considering the reservations made above, in connection with melting point variations of all-*trans*-γ-carotene samples, we have compared recently the bio-potency of pro-γ-carotene and its all-*trans* isomer obtained by iodine catalysis of the former. Both were found to have a provitamin activity of 41–42% of that of all-*trans*-β-carotene (Zechmeister, Pinckard, Greenberg, Straub, Fukui, and Deuel; unpublished).

We may also mention that the *Pyracantha* berries that contain substantial amounts of pro-γ-carotene are regularly consumed by some birds and mammals (porcupine) during the California season. These animals probably derive a portion of their A-requirement from this powerful provitamin.

4. *Cryptoxanthin Set*

The relative bio-potencies of all-*trans*-β-carotene and of its monohydroxy-derivative, all-*trans*-cryptoxanthin, were first determined by Kuhn and Grundmann (1934) who found that cryptoxanthin "caused in vitamin A-free rats in daily doses of 5 to 10 μg. about the same growth as β-carotene in 2.5 μg. doses." Their procedure had been described by Brockmann and Tecklenburg (1933). Considerably later Fraps and Kemmerer (1941) found that the bio-potency ratio of β-carotene and cryptoxanthin is 1.1:0.6, i.e., all-*trans*-cryptoxanthin shows an activity of 55%. Their experimental technique has not been reported in detail. Meanwhile, pertinent data seem also to have been published by Schormüller (1940) whose original contribution, however, has not been available to the present writer.

In order to coordinate the value of the ratio mentioned with stereochemical studies, a re-investigation was recently carried out in two independent series of bioassays which were made two months apart (Deuel *et al.*, 1945b). The resulting ratios were 100:54 and 100:59. We believe that the best available figure for the provitamin A potency of all-*trans*-cryptoxanthin is 57%, in good accordance with the experiments conducted by Fraps and Kemmerer (1941).

The main product of the *in vitro* isomerization of cryptoxanthin is neo-cryptoxanthin A (Zechmeister and Tuzson, 1938; Zechmeister and Lemmon, 1944) which should be interpreted as a central mono-*cis* form, probably 6-mono-*cis*-cryptoxanthin. Earlier authors mentioned only a single stereoisomer of cryptoxanthin (cf. Strain, 1938, 1941), termed "neo-cryptoxanthin" which was a mixture of much neo-cryptoxanthin A and but a little neo B. Their results are, therefore, directly comparable with ours. Fraps and Kemmerer (1941) gave as relative potencies, all-*trans*-cryptoxanthin: neo-cryptoxanthin = 0.6:0.4, which corresponds to 37% activity for neo-cryptoxanthin compared with that of all-*trans*-β-carotene. In recent bioassays 42% was found for neo-cryptoxanthin A (Deuel *et al.*, 1946).

Another member of this stereoisomeric set, viz., neo-cryptoxanthin U, a mono*cis* compound with a peripherally located *cis* double bond, was obtained recently in pure, crystalline form (Chatterjee and Zechmeister; unpublished). It shows a provitamin A activity in the rat of 27% of that of all-*trans*-β-carotene or 45% of that of all-*trans*-cryptoxanthin (Deuel, Greenberg, Straub, Fukui, Chatterjee, and Zechmeister; unpublished).

V. DISCUSSION OF BIOASSAY RESULTS

The data appearing in Table II illustrate the profound influence of the stereochemical configuration of a provitamin A on its bio-activity. Indeed, the order of magnitude of this influence is the same as that of some significant alterations in the chemical structure.

TABLE II

Relative Potencies of Stereoisomeric Provitamin A in the Rat

Stereoisomeric set	Stereochemical configuration (probable)	Current name	Bio-potency	
			in % of that of the all-*trans* form of the set	in % of the all-*trans*-β-carotene potency
α-Carotene	All-*trans*	α-Carotene	100	*53*
	Peripheral mono-*cis*	Neo-α-carotene U	25	*13*
	Central-peripheral di-*cis*	Neo-α-carotene B	30	*16*
β-Carotene	All-*trans*	β-Carotene	100	*100*
	Peripheral mono-*cis*	Neo-β-carotene U	38	*38*
	Central-peripheral-di-*cis*	Neo-β-carotene B	53	*53*
γ-Carotene	All-*trans*	γ-Carotene*	100	*27*
	All-*trans*	γ-Carotene**		*42*
	Peripheral mono-*cis*	Neo-γ-carotene P	70	*19*
	Central mono-*cis* etc.	(Mixture)	60	*16*
	About penta-*cis*	Pro-γ-carotene	160	*44*
Cryptoxanthin	All-*trans*	Cryptoxanthin	100	*57*
	Peripheral mono-*cis*	Neo-cryptoxanthin U	45	*27*
	Central mono-*cis*	Neo-cryptoxanthin A	74	*42*

* (*ex Pyracantha*).

** (*ex* pro-γ-carotene, by iodine catalysis).

For example, if we start in Table II from all-*trans*-β-carotene (100% effect) and convert it by moving out a double bond from the conjugated system, then this formation of all-*trans*-α-carotene reduces the potency to 53% of its initial value. However, the same extent of biological weakening can likewise be obtained without performing any chemical conversion, simply, by bending the all-*trans*-β-carotene molecule to give neo-β-carotene B (potency, 53%). Indeed, this decrease in the potency will be surpassed when we perform a single *trans* → *cis* rearrangement in a peripheral section of the all-*trans*-β-carotene molecule to produce neo-β-carotene U (potency, 38%). The latter decrease is even a little greater than that caused by the introduction of a hydroxyl group into the all-*trans*-β-carotene molecule which leads us to all-*trans*-cryptoxanthin (potencies, 100% → 57%).

A similar effect for neo-β-carotene B was observed by Johnson *et al.* in the chick which, on the basis of With's (1946a, b) important studies, is a satisfactory animal for the bioassay of provitamins A.

These examples show clearly that some chemical reactions which modify the position of a double bond or the status of functional groups, and some spatial rearrangements which modify the overall shape of a provitamin A molecule are of the same biological importance.

While one or two *trans*→*cis* rearrangements so far as we know, always decrease the bio-potency, this rule is not valid for poly*cis* compounds, since pro-γ-carotene and its all-*trans* stereoisomer have identical potencies in the rat. This was recently confirmed in unpublished experiments with chicks (Table III; Greenberg, Calbert, Pinckard, Deuel, and Zechmeister).

TABLE III

Relative Provitamin A Potencies of a Polycis-γ-carotene, viz., Pro-γ-carotene and Its All-trans Stereoisomer (All-trans-β-carotene = 100%)

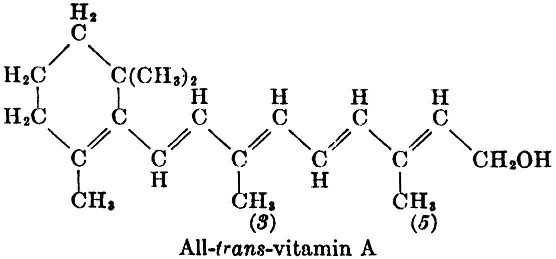
	Rat	Chick
Pro-γ-carotene	*41*	*51*
All-*trans*-γ-carotene (prepared from pro-γ-carotene by iodine catalysis)	*42*	*42*

It may not be a coincidence that the behavior of pro-γ-carotene is shown by a natural product, the molecules of which have an over-all rodlike shape and hence are morphologically closely related to all-*trans*-γ-carotene molecules.

It may be mentioned in this connection that the adsorption affinities observed *in vitro* are also influenced to about the same extent by some structural and by morphological variations respectively. A similar statement may be valid for the anchoring of provitamin A molecules in the living tissue.

VI. STEREOCHEMICAL ASPECTS OF THE CONVERSION PROVITAMINS A → VITAMINS A

According to the vitamin A structure as first given by Karrer *et al.* (1931), its molecule contains five double bonds. Of these, however, because of steric hindrance, probably only two will assume *cis* configuration, viz., the double bonds No. 3 and No. 5 (cf. the formula; and Pauling, 1939). Hence, vitamin A mainly occurs in the following four spatial forms:



All-*trans*-vitamin A
(Only the double bonds (3) and (5) are sterically unhindered.)
(In reality, the C═C—C angle is 125° 16′.)

All-*trans*-vitamin A (with some probability, ordinary vitamin A)
3-*cis*-vitamin A
5-*cis*-vitamin A
3,5-di-*cis*-vitamin A

Our knowledge in the field of stereoisomeric vitamins A is still very limited.

The so-called "allo"-vitamin A is not likely to be a member of this stereochemical set because of the position of its maxima and the sharp fine structure in the main spectral band of its ethyl ether (Milas *et al.*, 1948), while no fine structure is shown by a solution of crystalline vitamin A methyl ether (Hanze *et al.*, 1948).

Some earlier observations can be found in the literature which point to the occurrence of stereoisomeric vitamins A in nature. The presence of variable proportions of such isomers in some oils was predicted by Smith *et al.* (1938) and by Morton (1938). A little later Smith *et al.* (1939) reported that the exposure to ultraviolet radiation of A concentrates causes a marked decrease in the maximum extinction (328 mμ) which in the dark increases towards the initial value. In other instances a photochemical decrease was observed. Possibly, stereochemical changes also play a part in the observed initial increase in the fluorescence when alcoholic solutions of vitamin A esters are subjected to ultraviolet irradiation (Sobotka *et al.*, 1943).

A promising new development was made recently by Robeson and Baxter (1947) who succeeded in the isolation, from some fish liver oils, of a crystalline "neo-vitamin A" for which they tentatively suggest the 5-*cis* configuration. This would harmonize with a number of experimental data; however, a further explanation is needed of the fact that the position of the main spectral maximum of neo-vitamin A is located at a wavelength 3 mμ longer (instead of shorter) than the corresponding maximum in the ordinary vitamin A curve although the molecular extinction value for the neo-compound is somewhat lower than that for vitamin A.

Some characteristics of neo-vitamin A were also investigated by Meunier *et al.* (1948).

The bio-potency in the rat of Robeson and Baxter's neo-vitamin A was found to be identical with that of ordinary, crystalline vitamin A, as obtained by Baxter and Robeson (1942). This is in reasonable accordance with the following developments.

Recently, Cawley *et al.* (1948) reported that synthetic vitamin A concentrates also contain neo-vitamin A and that there the ratio of the two steroisomers (for example, vitamin:neo-vitamin = 2:1 or 1.5:1) is comparable to that in fish liver oil concentrates. Evidently, a catalytic interconversion, vitamin A $\rightleftharpoons$ neo-vitamin A, may take place both *in vitro* and *in vivo*. "It appears that 'vitamin A,' physiologically speaking, must be considered as a mixture of the two geometrical isomers." Whether or not the presence of the *cis* form is essential for the metabolism of the fish, remains undecided.

We proceed now to a discussion of vitamin A formation *in vivo*.

As is well known, β-carotene is converted into vitamin A in the animal body, according to the ideal formulation (Karrer *et al.*, 1931):

$$C_{40}H_{56} + 2\ HOH \rightarrow 2\ C_{20}H_{29}{\cdot}OH$$

In some instances and in the presence of sufficient tocopherol, this enzymatic cleavage was claimed to proceed quantitatively in the rat (Koehn, 1948). According to recent investigations, it takes place in the small intestine of this animal (Mattson, Mehl, and Deuel, 1947; Wiese *et al.*, 1947; Mattson, 1948; Thompson *et al.*, 1947; Glover *et al.* 1947).

It was suggested by Hunter (1946) that the reaction may not be a hydrolytic splitting of carotene *in vivo* but rather an oxidative cleavage to vitamin A-aldehyde which would then be subject to a reduction to vitamin A-alcohol:

$$C_{40}H_{56} \xrightarrow{+2\ O} 2\ C_{19}H_{27}{\cdot}CHO \xrightarrow{+4\ H} 2\ C_{19}H_{27}{\cdot}CH_2OH$$

Such a process would not biologically inactivate one half of the β-carotene molecule as considered by Koehn (1948).

Using hydrogen peroxide in the first step, the above-formulated conversion of β-carotene was successfully carried out *in vitro* although the yields were small (Hunter and Williams, 1945).

The possible contribution of such a mechanism to vitamin A formation *in vivo* would not alter our stereochemical discussion.

The picture does not seem to be complicated in mammals by *per se* vitamin A effects of carotenoids (i.e., without previous conversion to vitamin A), although, according to With (1946a, b) cryptoxanthin would act as such in the hen.

In the light of theoretical stereochemistry the direct conversion, provitamin A → vitamin A appears as follows.

Transition from the β-carotene set to the vitamin A set means a reduction of the number of the spatially probable configurations from 20 to 4. In other words, the twenty stereoisomeric β-carotenes must "converge" towards the four stereoisomeric vitamins A. Each one of the latter, of course, may then be subject to spatial and structural changes.

The hydrolytic cleavage of all-*trans*-β-carotene, yields, as is well known, two molecules of all-*trans*-vitamin A; however, the same product will be obtained from a β-carotene whose central double bond is in *cis* configuration, since the opening of this very bond destroys the only steric difference between all-*trans*- and this particular mono-*cis*-β-carotene. Hence, we may also expect that both all-*trans*-cryptoxanthin and neocryptoxanthin A will primarily yield identical conversion products in the rat.

It should also be mentioned that if a quasi-equilibrium mixture of stereoisomers, which is easily obtainable *in vitro*, could be quantitatively converted in the body to vitamin A (without previous steric rearrangement), then the product would consist preponderantly of all-*trans*-vitamin A molecules. For example, by refluxing β-carotene in petroleum ether a mixture composed as follows was obtained, unchanged all-*trans:* neo U:neo B:labile isomers = 86:4:8:2. Since both halves of the all-*trans*-β-carotene and one-half of neo B as well as of neo U yield all-*trans*-vitamin A, the molecules of the latter will be present to the extent of 92% (possibly a little more, if the "labile" isomers should also make a contribution). For iodine-catalyzed stereoisomeric β-carotene mixtures the corresponding figure would be about 87% all-*trans*-vitamin A (or a little higher).

VII. POSSIBLE MECHANISM OF THE *in vivo* CONVERSION STEREOISOMERIC PROVITAMINS A—VITAMINS A

The substantial differences in the bio-potencies of the respective spatial forms of a given provitamin A are, of course, the resultants of many partial bio-processes, the totality of which can be conveniently assayed by measuring the increase in weight of rats. Since neither the exact nature of these steps nor their dependence on the initial steric configuration is known, the present section offers an exposition of pertinent problems rather than their solution.

Some of the deciding factors which may play a part in these intricate processes are: the relative rates of absorption, the rates of addition of the respective provitamins to the enzyme system, and the rates of release of the emerging vitamin(s). The line which leads from the provitamin A content in a given diet to the vitamin A formed in the body may be further complicated by the unequal ease of deposition, destruction and excretion of the morphologically different provitamin A molecules.

In the interpretation of these processes it is a temptingly simple assumption to postulate that in the mammal body each *cis* provitamin A must first rearrange into its all-*trans* isomer before it can become a substrate for enzymatic cleavage. In other words, only the all-*trans* forms of α-carotene, β-carotene, γ-carotene, and cryptoxanthin would constitute true provitamins while any of their stereoisomers would be *per se* biologically inactive.

Although identical "digestibilities" of all-*trans*-β-carotene and neo-β-carotene B were found in the rat by Fraps and Meinke (1945a), stereoisomerization of *cis* provitamins can take place in the digestive tract according to Kemmerer and Fraps (1945a, c) who observed, after ingestion of neo-β-carotene U, that 45% of the crude carotene mixture derived from the feces was present in the all-*trans* form. Furthermore, after a Wesson oil solution of neo-β-carotene U had remained in the digestive tract for 6 hours, the extracted carotene mixture contained, beside neo-β-carotene U, 17.7% all-*trans*-β-carotene, 11.5% neo-β-carotene B, and 12.6% impurity "A." Since, however, the total of the carotenoids recovered in these experiments amounted only to one-fifth to one-eighth of the administered dose, one could not conclude from these data that the observed bio-activity of neo-β-carotene U is necessarily based on a primary rearrangement to all-*trans*-β-carotene.

That the all-*trans* configuration does not necessarily show maximum stability in the animal body, is also illustrated by an unpublished experiment in which pro-γ-carotene was fed to chicks. From the livers only a small fraction of the administered pigment could be recovered but this fraction consisted of the almost pure poly*cis* compound (Greenberg, Calbert, Pinckard, Deuel, and Zechmeister).

Another experimental approach to related problems was made recently by Johnson and Baumann (1947) who report that, in order to achieve the same vitamin A accumulation in rat liver and kidneys, very different daily doses of some stereoisomeric β-carotenes are required.

From this viewpoint the following doses were found to be biologically equivalent:

20 γ of all-*trans*-β-carotene,
40 γ of neo-β-carotene B,
60 γ of neo-β-carotene U, and
80 γ of all-*trans*-α-carotene.

On this basis, the relative A-accumulating potencies are, all-*trans*-β-carotene:neo-β-carotene B:neo-β-carotene U = 100:48:33 which is in agreement with the relative growth-promoting effects as established by our group, viz., 100:53:38. This remarkable parallelism still leaves, nevertheless, several interpretations open for the mechanism of the cleavage in the body; and it does not necessarily indicate a rearrangement of the two assayed *cis* compounds into all-*trans*-β-carotene previous to their cleavage.

At present, it would seem improbable to this writer that in the conversion to vitamin A the four all-*trans* provitamins should have absolute priority, in contrast to their allegedly biologically inert stereoisomers. We assume that the biological dividing line does not run between active *trans* compounds and inactive *cis* isomers but rather between such bioactive provitamin A molecules whose over-all shape does fit into the splitting enzyme system, and such inactive molecules that do not fit into the system, notwithstanding the presence or absence of *cis* double bonds. The poly-*cis* compound, pro-γ-carotene, may well be subject to direct conversion to vitamin A, considering the rodlike shape of its molecules, which are morphologically closely related to those of its all-*trans* isomer. It is significant that both are equally potent in the rat.

In summary, we may claim that, although only a limited progress has been made so far in the field under discussion, it has become increasingly evident that in suitable instances a stereochemical treatment may assist vitamin research and markedly broaden its scope.

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The Physiology of Thiamine

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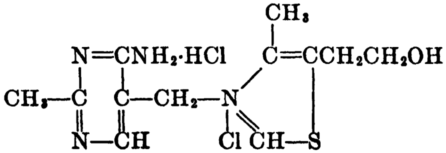
I. HISTORICAL INTRODUCTION

1. *General*

More than half a century ago Eijkman (1892) demonstrated that fowl fed polished rice developed a polyneuritis which soon ended fatally, or which could be cured by giving the animals raw rice. Several years later he proved (Eijkman and Vorderman, 1896) that beriberi, a deadly disease characterized by nerve lesions, was caused by eating polished rice as the chief food. Grijns, working in the same laboratory at Batavia (Java) demonstrated that polyneuritis in birds and beriberi in men, was caused by the lack of an extremely small quantity of an unknown substance. He called it "protective substance" (Grijns, 1900–1911). Funk gave it the name "vitamine." Later it was shown that several "vitamines" are not amines at all, and Drummond (1920) proposed that the final "e" be dropped.

Grijns and Funk tried to isolate the antineuritic or antiberiberi-vitamin, but isolation was not successful until 1926, when the present authors, (Jansen and Donath, 1926) succeeded in obtaining the substance in a pure, crystalline state. Again, it was not until a decade later that the structural formula was established and the synthesis was accomplished. (Williams and Cline, 1936; Grewe, 1936; Andersag and Westphal, 1937; Todd and Bergel, 1937).

The structural formula of the hydrochloric acid of thiamine appeared to be:



Thus forty years passed between the first indication of the existence of this substance and its synthesis in the laboratory. Once started, the industrial production proceeded astonishingly fast, and it was soon possible to deliver it in such quantities, and at such a low cost, that flour and rice for whole populations could be "enriched" with it.

2. *The Role of Thiamine in Tissue Metabolism*

Everyone who experiments on animals on thiamine deficient diets is struck by the fact that these animals lose weight every day, while their food-intake is reduced to a low level. After giving them a small fraction of a milligram of thiamine, however, their appetite is restored at once and their weight increases enormously. This led to the supposition that the vitamin has something to do with cell-metabolism. Several workers investigated the influence of thiamine on the metabolic rate of tissues. Westenbrink (1932a) has reviewed all the investigations published between 1920 and 1932 and analyzed the data from fifteen publications on this subject. About half the workers give a positive, and the other half a negative answer. However, after statistically evaluating the work, Westenbrink found that in only two of the fifteen investigations the metabolism of vitamin-rich tissues was significantly higher than in vitamin-poor ones. He himself could find no difference (1932b). The definite answer to this question came from the brilliant work of Peters and his school in Oxford. Peters realized that the development of opisthotonus, which is a sign of the last stages of polyneuritis in pigeons and fowls, proved that the disorder in thiamine-deficient animals affects both the peripheral and the central nervous system. In 1929 Peters and Kinnersley investigated the metabolism of polyneuritic pigeons; the only abnormality they could find was a slight increase in the amount of lactic acid in the brain of pigeons in opisthotonus. Peters also studied the oxygen-uptake by minced brain from pigeons in opisthotonus in comparison with that of normal birds. The great difference from many of his predecessors was that he added lactic acid (Kinnersley and Peters 1930) or in later experiments sugar or pyruvic acid to the brain (Peters and Sinclair 1933; Peters and Thompson 1934; Thompson 1934; Peters 1936). In a long series of experiments Peters demonstrated that thiamine plays a decisive part in carbohydrate, especially in pyruvic acid metabolism. Peters definitely established the fact that the metabolism of brain from polyneuritic pigeons, as measured by oxygen-uptake in Barcroft-Warburg tubes was less than that of the brain of normal pigeons. Furthermore, Peters demonstrated that the addition of a solution of thiamine to minced brain from polyneuritic pigeons suspended in pyruvic acid solution, increased the O_2 uptake of this mixture (catatorulin-effect of thiamine). This was the first instance of a chemical substance catalyzing an organ-tissue preparation (Passmore *et al.*, 1933). The work of the Oxford school was confirmed by Sherman and Elvehjem (1936). Further research led to the concept that the catalysis affecting the oxygen-uptake was not due to thiamine itself, but to a compound that was synthesized from the free vitamin (Peters *et al.*, 1935; Westenbrink and Polak, 1937). The nature of this compound became clear from another series of investigations. Neuberg and his collaborators (Neuberg and Karczag, 1911; Neuberg and Rosenthal, 1913) found an enzyme in yeast which decarboxylates pyruvic acid to acetaldehyde. They called it carboxylase. Simola (1932) showed that the tissues of rats on a thiamine-deficient diet had a greatly reduced content of carboxylase. Auhagen (1931, 1933) demonstrated that carboxylase could be rendered inactive by washing with a phosphate-buffer solution at pH 7–8. The activity was restored by adding an extract of boiled yeast. So he assumed that yeast contains a coenzyme, which he called cocarboxylase. Lohmann and Schuster (1937) succeeded in isolating this cocarboxylase in a pure, crystalline state, and they proved that it is the pyrophosphoric ester of thiamine. This coenzyme + alkali washed yeast + Mg ions decomposes pyruvic acid according to the reaction:

$$CH_3COCOOH \xrightarrow{\text{Carboxylase}} CH_3CHO + CO_2$$

Peters and his pupils demonstrated that the cocarboxylase is the active form of thiamine in tissue oxydation, also (Banga *et al.*, 1939a,b).

II. NOMENCLATURE

It took a long time after the discovery of the antiberiberi vitamin, to demonstrate that what in the beginning was called the water-soluble vitamin B, was not one factor, but that it consisted of two or more entities. These separate factors were distinguished as Vitamin B_1, B_2, B_3, etc. The vitamin B_1 was the special antiberiberi or antineuritic factor. The writer proposed the name "aneurine" for this substance (Jansen, 1935), a name which has been adopted fairly extensively in Europe. In America the name "thiamine" was introduced for the same substance. The physiologically active form, the pyrophosphate, was called aneurin pyrophosphate or thiamine pyrophosphate. In this article thiamine will be used in conformity with the policy of the editors of this publication. It is to be hoped that there soon will be one common nomenclature all over the world.

Many enzymes consist of a protein portion which is more or less firmly attached to a thermostable portion of lower molecular weight. In this article the nomenclature of Neuberg and von Euler is used and the combined enzyme will be referred to as the holoenzyme; the protein part as the apoenzyme; and the thermostable part as the coenzyme.

III. EXPERIMENTAL METHODS

The activity of thiamine has been studied by experiments on human beings, on animals (mammals, birds, and insects), on plants, and on microorganisms; also on animal tissues (slices, "brei" suspensions, dispersions, cell-free extracts) and finally on purified enzyme preparations. The tissue extracts or bacterial extracts may be obtained by grinding together with powdered glass or by ultrasonic waves (Stumpf, 1945).

Proof that thiamine pyrophosphate is responsible for a certain reaction, is usually obtained as follows: In higher animals the subject is put on a thiamine-deficient diet, so that the thiamine pyrophosphate content of the tissues diminishes markedly. In some organs (e.g., in liver) the depletion takes place in a short time, but in others (e.g., in heart and in nervous tissue) one must wait nearly until the life of the animal is endangered or until the symptoms of episthotonus appear (Barron *et al.* 1941a). The reactions of the whole depleted animal, or of its tissues (brain, liver, kidney, muscle, etc.) are then compared with those of normal animals. Eventually the effect of the addition of thiamine or of thiamine pyrophosphate is studied. When thiamine is added to tissues it must first be phosphorylated by incubating the tissues with it previous to the addition of the substrates (Barron *et al.*, 1941b).

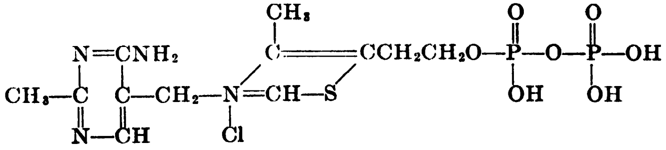
Microorganisms are grown in a thiamine-deficient medium, or the dried organisms (yeast, bacteria, etc.) are washed or dialyzed at a suitable pH to remove the thiamine pyrophosphate. In this case also it is often necessary to use cocarboxylase itself instead of thiamine, or to give additional triphosphopyridine nucleotide to phosphorylate the vitamin.

It is often difficult to obtain a diet or a culture medium that contains all essential nutrients except thiamine. In this respect, it was important that Eijkman, and Grijns, and other workers in the tropics used chickens or pigeons for their rice experiments, for polished rice lacks not only thiamine, but also several other nutrients. These other deficiencies of the rice are more serious than the lack of thiamine when rats and other mammals are used. In working with mammals the diet may be deficient in thiamine only. The pathological syndrome that is found in beriberi in men on a rice diet differed in several respects from the more or less pure thiamine deficiency as obtained (e.g., by R. D. Williams *et al.*, 1940, 1942) on a diet poor in thiamine, but otherwise well balanced.

Thiamine has a great influence on the appetite of men and animals (Cowgill, 1934). Thus, on a thiamine-deficient diet the food intake is severely reduced, and therefore it is often difficult to decide whether the reactions from the thiamine-deficient diet are due to lack of this vitamin or to general starvation. On the other hand the effects of a thiamine-poor diet may be masked by synthesis of the vitamin in the gut.

IV. CARBOXYLASE

Carboxylase is composed of a protein, the apocarboxylase and a prosthetic group, cocarboxylase: thiamine pyrophosphate. The pyrophosporic acid group is attached to the thiazole group of the thiamine molecule. Thus thiamine pyrophosphate has the formula:



The catalytic activity of thiamine pyrophosphate is of a high order. One mol of thiamine pyrophosphate working as a copyruvodehydrogenase in brain dispersions catalyses the uptake of 1500 mols of O_2 per minute (Banga *et al.*, 1939b). The activity of cocarboxylase in yeast is of the same order (Ochoa and Peters, 1938b). Monophosphothiamine has no activity as a coenzyme.

1. *Synthesis of Cocarboxylase*

Cocarboxylase can be synthesized from thiamine chemically or enzymically. However, because carboxylase is not dissociated (see below) the enzymic synthesis stops when the apoenzyme is saturated. No preparative use can therefore be made of this reaction.

a. Chemical Procedure. Lohmann and Schuster (1937) described a method for preparing the cocarboxylase. Weyland and Tauber (1938) gave an extensive description for the preparation of thiamine pyrophosphate which substance Weil-Malherbe (1940a) synthesized by treating the thiamine bromide with silver pyrophosphate. Karrer and Viscontini (1946b) improved the method of Weyland and Tauber. The strong hydrochloric acid that is present during the procedure of Weyland and Tauber (1938) tended to hydrolyze a large part of the thiamine pyrophosphate to monophosphothiamine. Therefore Karrer and Viscontini (1946b) improved the method by preparing the phosphate of the cocarboxylase. In this way they reduced the acidity of the solution and so obtained a yield that was 55% of the calculated one.

b. Enzymic Synthesis. Yeast, bacteria, and animal tissues contain systems that are able to phosphorylate thiamine. The first reports (Euler and Vestin, 1937; Lohmann and Schuster, 1937b) needed confirmation because thiamine strongly stimulates cocarboxylase activity (see Section IV, 2). Ochoa and Peters (1938a) and Ochoa (1939) studied the phosphorylation process in animal tissues. They found that liver shows a particularly good phosphorylating capacity. Slices, "brei" and "dispersions" were equally active. Brain and muscle were much less active; preparations from duodenal mucosa (pig) showed no activity at all. Ochoa thinks that the reaction

$$\text{Thiamine} + 2\ \text{Phosphate} \leftrightarrows \text{Thiamine Pyrophosphate}$$

is reversible. Oxygen is essential for a reaction to the right. The optimum pH is about 8.5. Goodhart and Sinclair (1939), working with blood from avitaminous pigeons demonstrated that the nucleated blood cells can phosphorylate thiamine.

A cell-free protein preparation containing the apocarboxylase has been made from yeast (Weil-Malherbe, 1939). He was able to demonstrate that phosphorylation of thiamine occurs only in the presence of adenylpyrophosphoric acid or such reactions as entail its intermediate formation.

Phosphopyruvic acid can act as a phosphate donor for synthesis of cocarboxylase in the presence of catalytic amounts of adenylic acid or thiamine pyrophosphate. Thus a direct transfer of phosphate from adenylpolyphosphate is the mechanism for phosphorylating thiamine. These results were confirmed by Lipton and Elvehjem (1940a) and by Quastel and Webley (1942). The synthesis does not take place via the monophosphate (Weil-Malherbe, 1939), for the production of thiamine pyrophosphate goes faster from thiamine itself than from its monophosphate. The synthesis of cocarboxylase is almost completely inhibited by 0.005 *M* iodoacetic acid, and only slightly inhibited by 0.04 *M* sodium fluoride (Lipton and Elvehjem, 1939). Westenbrink and his collaborators studied the synthesis of cocarboxylase by living yeast cells (Westenbrink *et al.*, 1947). By adding a large excess of thiamine to the yeast much more thiamine pyrophosphate is synthesized than can be bound to the apocarboxylase present in the yeast; it is probably bound to some other yeast protein. Contrary to the supposition of Sperber (1941), Westenbrink *et al.* (1947) proved that the synthesis of carboxylase by living yeast cells is not a simple reversion of the action of the phosphatase, the enzyme that decomposes the cocarboxylase.

2. The Dissociation of the Carboxylase

The bond between the apo- and the cocarboxylase at neutral or slightly acid pH is very firm. Washing with water and dialysis does not remove a trace of thiamine pyrophosphate. At pH about 8, the dissociation becomes total, so that by washing with an alkaline phosphate-buffer the thiamine pyrophosphate can readily be removed. Also at pH 4.6 the thiamine pyrophosphate may be split off (Stumpf, 1947). One exception, however, has been found in the enzyme that brings about the condensation of acetaldehyde (Green *et al.*, 1942) for the coenzyme is completely dissociated even in neutral solutions (Stumpf, 1945).

Stumpf assumes the thiamine pyrophosphate enzymes can be classified into two general groups depending on the relative degrees of dissociation. The first group, to which the majority of the known carboxylases, belong is not dissociated between pH 4.6 and 7.8. In the second group the enzyme is dissociated even in neutral solutions.

Ochoa and Peters (Ochoa, 1938) found that the addition of thiamine to apocarboxylase in the form of alkali washed yeast greatly enhanced the capacity of this apoenzyme for the production of CO_2 after addition of the coenzyme thiamine pyrophosphate and Mg. The activation was maximal with doses of thiamine twenty times larger than the thiamine pyrophosphate. Instead of thiamine itself also the pyrimidine-part of the molecule 2-methyl-4-aminopyrimidyl-5-methylaminodihydrochloride could be used (Ochoa and Peters, 1938b). This proved that the activation is not caused by synthesis of the added thiamine to thiamine pyrophosphate. This stimulation by thiamine of the resynthesis of thiamine pyrophosphate was much greater in bakers' than in brewers' yeast (Lipton and Elvehjem, 1940b). Westenbrink and his collaborators succeeded in giving an explanation for this activation (Westenbrink and van Dorp, 1940; Westenbrink *et al.*, 1940a). They found a powerful phosphatase in yeast; this phosphatase is capable of hydrolyzing the phosphoric esters of thiamine in a very short time. The thiamine does not actually stimulate the carboxylase system, but only inhibits the phosphatase that destroys the carboxylase.

When the coenzyme once is bound to the protein, thiamine pyrophosphate is resistent to this phosphatase (Westenbrink *et al.*, 1940b). This is in accordance with the observation of Lipmann (1939a), confirmed by Lipton and Elvehjem (1940b) and by Westenbrink, that thiamine is without any effect when it is added to the yeast suspension some minutes after the addition of cocarboxylase, for then the cocarboxylase is destroyed by the phosphatase. Westenbrink *et al.* (1941) demonstrated the high enzymic activity of this phosphatase: in 10 seconds it dephosphorylated a large part of the cocarboxylase. The objection of Lipton and Elvehjem (1940b) that phosphate, the other product of the splitting of thiamine pyrophosphate did not inhibit the phosphatase-action on cocarboxylase could be answered by the assumption that in this case there is no shift of the equilibrium according to the law of mass action, but a competitive inhibition. It appeared later that animal phosphatases are not inhibited by thiamine, but by phosphate ions (Westenbrink *et al.*, 1943a).

The synthesis of carboxylase by bringing together alkali-washed yeast, magnesium, and thiamine pyrophosphate was thoroughly studied by Parvé (1945) (see also Section V, 3b). Even when a large amount of thiamine is added a great deal of the thiamine pyrophosphate is hydrolyzed before it is bound to the protein and becomes resistent to the phosphatase. Therefore, only part of the thiamine pyrophosphate is resynthesized to carboxylase; at most about 25%.

In all respects, the effect of washing at pH 6.2 or 5.6, the resynthesis at pH 6.8, the dissociation at pH 7.8 and the activity per gamma of thiamine pyrophosphate, the resynthesized carboxylase was equally as stable as the original holoferment. Only one difference was found. The maximum activity, measured by the production of CO_2 from pyruvate, is about pH 5.7 for the native carboxylase and pH 6.2 for a mixture of alkali-washed yeast + magnesium ions + thiamine pyrophosphate. This difference is easily explained for the optimum pH for the resynthesis is 6.8. In the mixture of alkali-washed yeast + coferment two reactions are going on simultaneously: the synthesis of the holoenzyme and the action of this enzyme on pyruvate. So pH 6.2 is a compromise between pH 5.7 and 6.8.

Lipton and Elvehjem (1940b) gave another explanation for the stimulation of the formation of carboxylase by thiamine; they postulated that a substance in yeast, probably a protein other than the enzyme protein, adsorbs the cocarboxylase and in this way inhibits the formation of the holoenzyme carboxylase. The addition of excess thiamine saturates this material and thus permits the adsorption of the thiamine pyrophosphate on the apoenzyme.

Most of their experiments could be interpreted just as well by the supposition of Westenbrink, and van Dorp (1940), as by that of Lipton and Elvehjem. There was one experiment that was easier explained by the Wisconsin than by the Amsterdam investigators: Lipton and Elvehjem demonstrated that by incubating the apoenzyme with cocarboxylase at 30°, the recovery of carboxylase gradually increased as the incubation period increased. However, Westenbrink and his collaborators could not confirm these results with the kind of yeast they had at their disposal.

Buchman *et al.* (1940) found a competitive inhibition of thiamine pyrophosphate by the thiazole pyrophosphate portion of the cocarboxylase molecule. Therefore they assumed that the thiamine pyrophosphate is bound to the apoenzyme through the pyrophosphate group which is common to both the cocarboxylase and the inhibitor.

V. CATALYTIC ACTIVITIES OF THIAMINE PYROPHOSPHATE ENZYMES

We have seen that thiamine pyrophosphate is the form in which thiamine is catalytically active. We do not yet know the biological processes in which thiamine or some compound other than cocarboxylase, is working. Perhaps thiamine itself together with thiamine pyrophosphate plays a role in nerve activity (see von Muralt, 1947).

The many reactions catalyzed by enzyme preparations from animal tissues, yeast, and bacteria in which thiamine pyrophosphate is the coenzyme are listed in Table I.

Table I indicates that many different reactions are catalyzed by these enzymes, and that most of them attack pyruvic acid or other α-keto fatty acids. It may be remarked that pyruvic acid stands at the crossroads of the carbohydrate metabolism; it is the end point of the anaerobic processes, and the starting point of the decarboxylation and oxidation

TABLE I

Reactions Catalyzed by Thiamine Pyrophosphate Enzymes

Reactions	References
Pyruvic acid → Acetaldehyde + CO_2	Lohmann and Schuster, 1937; Kensler *et al.*, 1942; Cajori, 1942.
Pyruvic acid + Oxygen → CO_2 + H_2O	Peters and his school; see Peters, 1946; Stumpf *et al.*, 1947.
Pyruvic acid + Oxygen → Acetic acid + CO_2	Lipmann, 1937; Still 1941; Stumpf, 1945; Stumpf *et al.*, 1947.
Pyruvic acid + Phosphate + Oxygen ⇄ Acetylphosphate + CO_2 + H_2O	Lipmann, 1944.
Pyruvic acid + Phosphate → Acetylphosphate + H_2 + CO_2	Utter and Werkman, 1943; Kalnitzky and Werkman, 1943.
Pyruvic acid + Phosphate → Acetylphosphate + HCOOH	
Pyruvic acid + CO_2 → Oxalacetic acid	Krebs and Eggleston, 1940; Smyth, 1940
Pyruvic acid + Oxygen → Citric acid	Sober *et al.*, 1940; Barron *et al.*, 1941.
Pyruvic acid + Oxygen → Acetoactic acid	Barron *et al.*, 1941a.
Pyruvic acid + Oxygen → α-Ketoglutaric acid	
Pyruvic acid + Oxygen → Succinic acid	
Pyruvic acid + (Oxygen) → Carbohydrate	
Pyruvic acid → Formation of Acetylcholine	Mann and Quastel, 1940; Minz, 1946.
Pyruvic acid → Acetoin + CO_2	Silverman and Werkman, 1941; Green *et al.*, 1941, 1942; Cajori, 1942.
Acetaldehyde → Acetoin	Green *et al.*, 1942.
Acetic acid + Oxygen → CO_2 + H_2O	Quastel and Webley, 1941, 1942.
α-Ketobutyric acid + Oxygen → Propionic acid + CO_2	Long and Peters, 1939; Stumpf, 1945.
α-Ketovaleric acid + Oxygen → Butyric acid + CO_2	Long and Peters, 1939.
α-Ketobutyric acid → Propioin + CO_2	Green *et al.*, 1942.
α-Ketoglutaric acid → Succinic semialdehyde + CO_2	Green *et al.*, 1941, 1942.
α-Ketoglutaric acid + Oxygen → Succinic acid	Stumpf *et al.*, 1947, Barron *et al.*, 1941b.
2-Diacetyl + $2H_2O$ → Acetoin + 2 Acetic acid	Green *et al.*, 1947.

reactions and of the reversal of the anaerobic chain of reactions. This attack on pyruvic acid may be a simple decarboxylation, an oxidative decarboxylation, a CO_2 fixation, a dismutation, or a condensation. We may assume that the exact type of reaction depends upon the protein, the apoenzyme, with which the thiamine pyrophosphate is combined.

In the experiments with tissues or crude extracts, the enzyme system contained several other enzymes and catalytic substances, such as the C_4

dicarboxylic acids, besides the carboxylase. In their initial work Long and Peters (1939) concluded that there is a distinct difference between the systems in yeast and the animal tissue. The yeast system decarboxylates only the pyruvic acid, whereas in the tissue oxidation accompanies decarboxylation. Animal tissues contain a pyruvic acid dehydrogenase. From these results they inferred that the apoenzymes are different in yeast and in animal tissues.

Thus, thiamine pyrophosphate can act in yeast as the coferment of pure carboxylase, whereas in animal tissues and in several bacteria it acts as the coferment of a pyruvic dehydrogenase.

a. The Unitarian View. Coenzymes commonly catalyse different reactions, depending upon the nature of the apoenzyme. However, the difficulty with thiamine pyrophosphate is that the two kinds of reactions are so widely different from a chemical point of view, one is oxidative and the other is nonoxidative. Also, none of the mechanisms proposed for the oxidative action of thiamine pyrophosphate has proved to be satisfactory (see Section V, 1c). Krebs drew attention to this difficulty, and he was the first who advanced an attractive unitarian theory, bringing all the different activities of thiamine under one single denominator. Evans, working in Krebs' laboratory (Evans, 1940) had shown that minced pigeon liver was able to oxidize pyruvate to α-keto glutarate. From experiments to elucidate the mechanism of this reaction, Krebs and Eggleston (1940), were led to the assumption that the primary step is an assimilation of carbon dioxide to pyruvic acid, a Wood and Werkman reaction (1936, 1938):

$$CO_2 + CH_3COCOOH \rightarrow COOH{\cdot}CH_2CO{\cdot}COOH$$

The oxaloacetate formed in this way is converted into citrate and α-keto glutarate according to Krebs' citric acid cycle. Krebs and Eggleston assumed that the first of this cycle of reactions, the formation of oxaloacetate, (the reverse of "decarboxylation") is catalysed by thiamine pyrophosphate. In this way, the vitamin is not directly concerned with the oxidation of pyruvate, but with a reaction preliminary to the oxidation; the oxaloacetate acts as a hydrogen carrier. However, the experimental evidence for this theory was not very convincing. Their chief argument was based on the demonstration with suspensions of pigeon liver that pyruvate utilization was greatly increased by suspending it in bicarbonate solution instead of in phosphate buffer, and by adding CO_2 to the gas phase; whereas CO_2 and bicarbonate have no general effect on processes in liver suspensions.

Krebs also demonstrated that acetoacetate formation is somewhat reduced in livers of thiamine-deficient pigeons and that addition of the vitamin restored this formation.

Furthermore Smyth (1940) as collaborator of Krebs, prepared cultures of *Staphylococcus aureus* or *S. albus*, deficient in thiamine. The pyruvate metabolism of these microorganisms was much lower than that of vitamin-saturated cells. Added thiamine greatly enhanced this pyruvate metabolism. In this respect thiamine could be replaced by oxaloacetate. This effect could not be obtained in Staphylococci grown in vitamin-sufficient media. So these experiments are in accordance with the assumption that thiamine catalyzes the formation of oxaloacetate, and that this substance acts as a hydrogen carrier in the dismutation of pyruvate. At the end of his article Krebs drew attention to the work of Ruben and Kamen (1940) who were able to demonstrate, with the aid of radioactive carbon, that animal tissues were able to assimilate CO_2. In the following years several investigators firmly established the fact of the assimilation of carbon dioxide by animal tissues (e.g., Evans and Slotin, 1941; Solomon *et al.*, 1941; Utter and Wood, 1946). However, the role of thiamine pyrophosphate in this process is not definitely proven, and there are several experimental data, which cannot be brought in line with this unitarian view of Krebs. Barron *et al.* (1941b) in contradiction to the results of Smyth with bacteria, stated that the condensation reactions of pyruvate in tissues of avitaminotic animals were accelerated by thiamine but not by oxalacetate. The results of Green with the purified carboxylase preparations are not easily reconciled with the Krebs' theory.

b. The Coupling of Pyruvic Acid Oxidation with Phosphorylation. The coupling of oxidations with phosphorylations with high-energy and low-energy phosphate bonds, cuts the flow of energy of the oxidation into fractions (Lipmann 1941). In this way it might be thought that the difference between oxidizing and nonoxidizing enzymes becomes less important. Lipmann (1937, 1939b) studied the oxidation of pyruvate by an extract from *Bacillus delbrückii* (*Bact. acidificans longissimum*). The pyruvic acid is oxidized to acetic acid and CO_2:

$$CH_3COCOOH + \tfrac{1}{2}O_2 \rightarrow CH_3COOH + CO_2$$

The reaction requires inorganic phosphate and adenylic acid. The inorganic phosphate disappears during the process and an equivalent amount of adenosine polyphosphate is formed. Thus the oxidation generates an energy-rich phosphate bond. The primary oxidation product of the pyruvic acid behaves like acetylphosphate. Synthetic acetylphosphate is able to transfer its high energy phosphate bond to adenylic acid. From these facts Lipmann formulated the oxidation process:

$$CH_3COCOOH + H_3PO_4 + O_2 \rightarrow CH_3COOPO_3H_2 + CO_2 + H_2O_2.$$

Afterwards Lipmann (1944) calculated the bond energy of acetyl phosphate. As it is some 3 kcal. higher than the average energy of 12 kcal. of the energy-rich bond, it will amount to around 15 kcal. The need of inorganic phosphate for pyruvate oxidation in brain also was demonstrated (Banga *et al.*, 1939a, c). However Ochoa *et al.* (1939) could find no indication that acetylphosphate is an intermediate in the oxidation of pyruvic acid by brain or by muscle. Thus there are many carboxylases that require inorganic phosphate for their action. However, there are exceptions to this rule: Stumpf (1945) studied the pyruvic acid oxidation by *Proteus vulgaris*, which contains an enzyme that, in the presence of thiamine pyrophosphate and magnesium, specifically catalyzes the oxidative decarboxylation of pyruvic acid to acetic acid and CO_2 with liberation of considerable free energy:

$$CH_3COCOOH + \tfrac{1}{2}O_2 \rightarrow CH_3COOH + CO_2.$$

No disappearance of pyruvic acid could be observed under anaerobic conditions and no CO_2 was produced. This enzyme from *Proteus vulgaris* is equally active in the absence, as in the presence, of inorganic phosphate. In several ways Stumpf could demonstrate that the assumption that trace amounts of phosphate should react with pyruvic acid to form acetylphosphate, which could then act as a catalyst, was not in accordance with the facts. Similarly the pyruvic oxidase of *Escherichia coli* (Still, 1941) does not require the presence of inorganic phosphate. Ochoa (1941) thoroughly studied the coupling of phosphorylation with oxidation of pyruvic acid in the brain and the oxidation of the α-ketoglutaric acid (1944) by a preparation from the heart of the cat. He confirms the observation that inorganic phosphate and adenylic acid or adenine triphosphate are required for the oxidation. The rate of oxygen uptake by the dehydrogenase from cat-heart depended upon the concentration of inorganic phosphate. However, it is not certain that this preparation contained a thiamine pyrophosphate enzyme.

Kalnitzky and Werkman (1943), working with extracts of *Escherichia coli* found that inorganic phosphate was essential for the activity of its pyruvic acid hydrogenase. Utter and Werkman (1943) showed that the apparently "hydroclastic" action of this extract: $CH_3COCOOH + H_2O \rightarrow CH_3COOH + HCOOH$, was really a "phosphoroclastic" split. Utter *et al.* (1944) with the aid of C^{13} as a tracer demonstrated that this reaction:

$$CH_3COCOOH + H_3PO_4 \rightleftarrows CH_3COO(PO_3H_2) + HCOOH$$

is reversible.

Acetic acid is formed by the dephosphorylation of acetylphosphate.

This reversibility shows that a thiamine pyrophosphate enzyme can catalyze a synthesis, as well as a breakdown, of pyruvate.

c. The Proposed Mechanisms of the Oxidative Action. Langenbeck (1933) made model experiments on enzyme reactions. Referring to these experiments, Weil-Malherbe (1940b) thinks that a Schiff base is first formed and that this base undergoes an intramolecular oxidation-reduction process. No experimental evidence was given for this view. On the contrary Stern and Melnick (1939) presented experimental data against the Langenbeck-cycle.

Lipmann (1936) reduced thiamine with platinum black and H_2 or by $Na_2S_2O_4$. He assumed that a dehydro derivative might be formed which forms a redox system with the vitamin. In 1938, however, Lipmann and Perlman showed that the activity of thiamine was lost after dehydrogenation. Stern and Melnick (1939) pointed out that the sulfite, formed in the reaction of thiamine with sodium dithionite, must split the vitamin molecule (Williams *et al.*, 1935). This was proven by Karrer *et al.* (1945); Karrer and Viscontini (1946a). Furthermore, Stern and Melnick (1939) showed that reduction of thiamine with activated hydrogen gave a product that was not autoxidizable, and was biologically inactive when tested on polyneuritic pigeons. Cautious reduction of thiamine pyrophosphate with activated hydrogen, however, gave a dihydrococarboxylase that also was not autoxidizable, but that was active in curing polyneuritic pigeons.

On the other hand Zima and Williams (1940) and Zima *et al.* (1941) oxidized the sodium salt of thiamine with iodine and obtained a product which they assumed was formed from two molecules of thiamine, in which the S-H of the thiazole half of the molecule is transformed to S-S. Thus, it appeared that the thiamine/thiamine disulfide should form a reduction-oxidation system just as cysteine/cystine or glutathione and its disulfide.

Karrer and Viscontini (1946a, 1946b) however, chemically synthesized the thiamine-disulfide-pyrophosphate. This was combined with the apoenzyme, the alkali-washed yeast. The product was inactive with pyruvic acid. Therefore the disulfide form of cocarboxylase is not the active coenzyme. Nevertheless, the disulfide may be active in the animal organism, but only by preliminary reduction to thiamine itself. Similarly, Peters (1946b) found that the disulfide is active only after reduction. But the fact that *in vitro* tissue preparations are able to reduce the disulfide appears, in Peter's opinion, to leave room for the suggestion of Williams and Zima, that the disulfide may play a part in the dehydrogenation.

Barron and Lyman (1941) showed that the thiamine pyrophosphate was more resistant to oxidation and reduction than thiamine itself. They concluded that the action of thiamine pyrophosphate as a component of enzyme systems does not involve reversible oxidation and reduction. They were led to postulate that the phosphorylated thiamine acts as the integral part of the activating protein. After summarizing all the work that has been performed until now, we must conclude that much more experimental work is necessary, preferably with pure pyruvodehydrogenase preparations, to establish the mechanism for the oxidative activity of thiamine pyrophosphate.

2. The Essential Metals

Most of the carboxylases are thiamine pyrophosphate-magnesium-protein compounds (Ochoa, 1939). Magnesium may be replaced by manganese. Green, D. E. *et al.* (1941), using a preparation from top brewer's yeast, made an elaborate study of the replacement of magnesium by other metals. All the divalent metals tested could replace magnesium. The trivalent form of iron and the trivalent aluminum were practically inactive (Table II).

TABLE II

Evolution of CO_2 from Pyruvic Acid by Thiamine Pyrophosphate Protein and Different Metals

Metal, 1 mg. per 3.3 cc.	No addition	Mg^{++}	Mn^{++}	Co^{++}	Cd^{++}	Zn^{++}	Ca^{++}	Fe^{++}	Al^{+++}	Fe^{+++}
CO_2, mm.^{3}	23	875	850	694	422	282	558	550	50	39

Stumpf (1945) in his pyruvic oxidase from *Proteus vulgaris* replaced the Mg by Mn″, Fe″, Ni″, Zn″ or Co″, but Ca″, Ba″, Cd″ and the trivalent Fe and Al″″ were ineffective. Green *et al.* (1947) stated that their preparation of diacetyl mutase from pigeon breast muscle did not require magnesium or any other divalent metal.

3. Specificity

a. As Regards Thiamine Pyrophosphate. In pure preparations not containing a phosphorylating system, thiamine pyrophosphate cannot be replaced by thiamine, monophosphothiamine, pyrithiamine (Stumpf, 1945), diphosphopyridine nucleotide, triphosphopyridine nucleotide, flavine dinucleotide, adenosine triphosphate, or pyridoxal phosphate (Green *et al.*, 1947).

b. Substrate Specificity. Green, D. E. *et al.* (1941) studied the activity of a purified carboxylase from yeast on different substrates (Table III). Thus, the α-ketonic acids, in addition to pyruvic acid, are decarboxylated also, but the higher homologues are attacked at a lower rate.

TABLE III

Decarboxylation of α-Ketonic Acids by Yeast Carboxylase

Substrate 0.04 *M*	Mm.^{3} CO_2
Pyruvic acid	596
α-Ketoisovaleric acid	525
Oxaloacetic acid	191
α-Ketoisocaproic acid	24
α-Ketoglutaric acid	8
Phenylpyruvic acid	0
α-Hydroxyphenylpyruvic acid	0
Acetoacetic acid	0
No addition	0

The specificity of carboxylases from animal tissues was somewhat different (Green, D. E. *et al.*, 1942). These preparations had no action on oxaloacetic acid, mesoxalic acid, α-ketocaproic acid or phenylpyruvic acid. They decarboxylated α-ketobutyric acid under formation of propioin, according to the equation:

$$2\ CH_3{\cdot}CH_2{\cdot}CO{\cdot}COOH \rightarrow CH_3CH_2{\cdot}CHOH{\cdot}CO{\cdot}CH_2CH_3 + 2\ CO_2;$$

and α-ketoglutaric acid to succinic semialdehyde and CO_2:

$$COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}COOH \rightarrow COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CHO + CO_2.$$

The decarboxylation of α-ketoglutaric acid took place with the same velocity as that of pyruvic acid. Green *et al.* made it probable that the pyruvo (apo) carboxylase and the α-ketoglutaro (apo) carboxylase were different enzymes. For if thiamine pyrophosphate was split from the carboxylase by washing at pH 4.6, the maximum rate for CO_2 development with pyruvate as a substrate was found by the addition of a 2 γ of thiamine pyrophosphate per cubic centimeter of Green's preparation. With α-ketoglutarate as a substrate, the maximum reaction velocity was attained only when 300 γ of thiamine pyrophosphate per cubic centimeter was added. A third enzyme seemed to be present in Green's preparation, an enzyme for the condensation of acetaldehyde to acetoin. For maximum activity it required 30 γ of thiamine pyrophosphate per cubic centimeter. Each enzyme perhaps has a very high specificity for different substrates and may be catalyzed by different apoenzymes plus the same co-enzyme.

The pyruvic oxidase, prepared by Stumpf (1945) from *Proteus vulgaris* proved to be very specific. It oxidizes only pyruvic acid; α-ketobutyric, α-ketocarpoic, α-ketoglutaric, acetoacetic, glyoxylic and lactic acids are not attacked. The enzyme preparation oxidized formic acid and phenylpyruvic acids, even in the absence of thiamine pyrophosphate. Therefore, the oxidation of these two acids cannot be attributed to the thiamine pyrophosphate-pyruvic acid oxidase. The diacetyl mutase preparation of Green *et al.* (1947) from animal tissues *was* found to be highly specific for diacetyl. Glyoxal, methylglyoxal, dipropionyl, benzil, cyclohexamedione -1, 2, alloxan, and cyclohexanehexone were not attacked by the enzyme. However, the same preparation catalyzes the oxidative decarboxylation of pyruvic acid and of α-ketoglutaric acid (Stumpf *et al.*, 1947). Here again, the α-ketoglutaric oxidase, after splitting off of the thiamine pyrophosphate required a larger amount of thiamine pyrophosphate than the pyruvic oxidase. Here they found still more cogent evidence bearing on the nonidentity of the pyruvic acid and the α-ketoglutaric oxidase from summation experiments. For example, with concentrations of substrates that were adequate to saturate the respective enzymes, α-ketoglutaric acid was oxidized at the rate of 59 mm.^{3} of O_2 per 30 minutes, whereas the rate for pyruvic acid was 37 mm.^{3} in the same period. The rate with both substrates together was 92 mm.^{3}, which represents a virtual summation of the two rates. Were a single enzyme involved, the rate in the presence of both substrates would be expected to be in between the two rates, but never greater than the rate of either.

4. Sedimentation in Strong Centrifugal Fields

Most of the preparations from animal sources are easily sedimented, e.g., by centrifuging 30 minutes at 15,000 or 20,000 R.P.M. (Ochoa, 1944; Green *et al.*, 1947). Some of the enzyme preparations from bacteria seem to be more resistant to centrifugal force. Thus Silverman and Werkman (1941) state that the carboxylase from *Aerobacter aerogenes* remained in the supernatant solution after centrifuging for 1 hour at 250,000–300,000 R.P.M. With a preparation from *Proteus vulgaris*, Stumpf (1945) found that centrifugation for 1 hour in a gravitational field of 9,700 G sedimented only a small part of enzyme. However, centrifugation for 1 hour in a gravitational field of 100,000 G sedimented all active material. There seems to be a difference between the different enzymes.

5. Inhibitors and Activators

Most of the preparations show no inhibition by $3.3 - 10 \times 10^{-3}$ *M* iodoacetate, fluoroacetate, fluoride, malonate (Green, D. E. *et al.*, 1941; Green *et al.*, 1942; Stumpf, 1945; Stumpf *et al.*, 1947). Some preparations are more or less inhibited by iodoacetate and fluoride. Thus the enzyme from *Aerobacter aerogenes* is inhibited 85% by 7×10^{-3} *M* iodoacetate, and 36% by 5×10^{-3} *M* fluoride (Silverman and Werkman, 1941); the diacetyl mutase from pigeon breast muscle was inhibited 39% by 3.3×10^{-3} *M* iodoacetate (Green *et al.*, 1947).

Salts of heavy metals were found to be strong inhibitors for the cocarboxylase from yeast (Green, D. E. *et al.*, 1941). A *M*/10,000 concentration of $AgNO_3$, $CuSO_4$ or $Hg(NO_3)$ gave complete inhibition, but *M*/10,000 $ZnSO_4$ showed no inhibition at all.

Several other inhibitors are described for carboxylases. Cajori (1942) found inhibition for carboxylase from yeast by hemin; the enzyme was activated by cystein. Kensler *et al.* (1942) showed inhibition of yeast carboxylase by the split products of *N*-*N*-dimethylaminobenzene; they confirmed the stimulation by cystein.

Stumpf (1945) working with a pyruvic oxidase from *Proteus vulgaris* found that inorganic pyrophosphate in 3.4×10^{-3} *M* concentration inhibits the enzyme activity; this inhibition is reversed by 0.7×10^{-3} *M* thiamine pyrophosphate. He ascribed this inhibition by inorganic pyrophosphate to its reversible union with the magnesium-protein complex, thus blocking a similar union with thiamine pyrophosphate. See also Sections IX and XI, 3.

6. *Summary*

In our present concept, the metabolic enzymes are composed of a protein part, an apoenzyme, and a coenzyme. The coenzyme determines the group specifity of the enzyme activity; the finer specificity depends on the apoenzyme. The thiamine pyrophosphate enzymes, with their widely different actions, present a nice example of this concept. The difficulty in case of the thiamine pyrophosphate enzyme is that they have both an oxidative and a nonoxidative activity. In the older experiments with tissues and crude tissue extracts, it is likely that the oxidative reactions were catalyzed by other enzymes that were present. But even the more purified preparations of Green and his school that were free from other coenzymes (adenosine triphosphate, pyridoxalphosphate, cytochrome C, or flavine-adenine-dinucleotide) showed an oxidative and a nonoxidative activity (see e.g., Green *et al.*, 1947 and Stumpf *et al.*, 1947). Furthermore, the proposed mechanism of the oxidative action of thiamine pyrophosphate is not satisfactory. Thus, it remains for future research to clear up these difficulties.

VI. THE ROLE OF THIAMINE IN PROTEIN-METABOLISM

The enzyme that is active in the decarboxylation and transamination of amino acids, does not contain thiamine, but another vitamin of the B group, vitamin B_6, or pyridoxine. So it is usually taken for granted that thiamine does not play a role in protein metabolism. Braunstein

(1947) particularly reviewed the Russian publications which indicate that thiamine has something to do (directly or indirectly?) with protein metabolism. Thus he reports a more or less specific pathological disturbance of transamination in the form of a marked decrease in glutamic aminopherase and aspartic aminopherase activity in all tissues of thiamine deficient pigs and rats, described by Kritzmann (1940, 1943). These effects were absent in starved controls and the aminopherase activity was rapidly restored upon administration of thiamine. However, the original publications of Kritzmann are not available to the writer, and he is not able to judge their value.

The fact that Dann (1945) was able to maintain rats for more than a year on a thiamine-free synthetic diet, containing 80% purified casein, indicates that thiamine may not be essential for the metabolism of proteins.

VII. The Role of Thiamine in the Metabolism of Fats

1. The Thiamine Sparing Action of Fats

Evans and Lepkovsky (1928, 1929) showed that a diet containing 50% lard, but no antineuritic vitamin, did not give rise to the known deficiency symptoms. So the fat had a "sparing" action on the thiamine. This thiamine sparing action of fats was confirmed by several authors in different ways (Gregory and Drummond, 1932; Lecoq, 1932; Westenbrink, 1934; Stirn *et al.*, 1939; MacDonald and McHenry, 1940; Banerji, 1940). The most obvious explanation of this sparing action is the assumption that thiamine is not a constituent of any enzyme system active in the metabolism of fats.

However, part of the thiamine sparing activity of fats may be ascribed to the bacterial synthesis of thiamine in the gut of rats on diet rich in fats (Whipple and Church, 1935).

2. Transformation of Carbohydrates into Fats

It is known that the animal organism can synthesize saturated fatty acids from carbohydrate. As the pyruvic acid has a key position in the metabolism of carbohydrates, and thiamine plays a role especially in the metabolism of this intermediate, it would be expected that thiamine is active in the process of building fats from carbohydrates.

The literature until 1944 on the influence of vitamins on the synthesis of fats from carbohydrates has been reviewed by McHenry and Cornett (1944). From this review it is clear that the older publications strongly support the view that vitamins, particularly thiamine, play a significant role in the transformation of carbohydrates into fats. Boxer and Stetten

(1944), however, in paired feeding experiments using deuterium as a tracer, could not find any difference in the deposition of newly synthesized fats in rats that were fed on a complete diet as compared to rats on a thiamine-free diet, provided that the food intake in both cases is the same. From these experiments they concluded that a supply of thiamine is a necessary, but not sole condition for the formation of fats from carbohydrates. (See also Stetten and Boxer, 1947.)

VIII. THE COURSE OF THIAMINE IN THE ANIMAL ORGANISM

Thiamine is synthesized by plants, and also by certain microorganisms. Other microorganisms are able to synthesize thiamine from its pyrimidine and thiazole moieties (see Schopfer, 1939). Abderhalden and Abderhalden (1939) stated that tissue extracts from higher animals are capable of synthesizing thiamine from a mixture of the pyrimidine and thiazole fractions. However, this was true only to a slight extent for about 1% of the theoretical amount was formed. Abderhalden (1940) protected pigeons and rats on a thiamine-free diet from deficiency symptoms by giving the animals large quantities of the pyrimidine and the thiazole moieties.

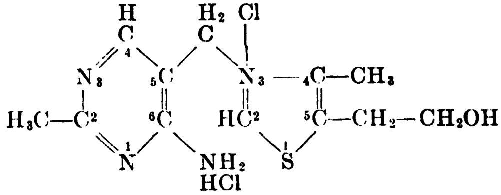
Furthermore the microorganisms in the gut are able to synthesize a small amount of thiamine; under certain circumstances all the thiamine that is necessary for the animal can be synthesized in the intestines. Fridericia *et al.* (1928) called this phenomenon "refection." This synthesis by intestinal bacteria plays an important role, particularly in ruminants (Theiler *et al.*, 1915).

In general, however, the source of thiamine for men and animals is the food they eat. Most of the thiamine in plants is present as the free vitamin. Thus, in cereals there is practically no pyrophosphate, in potatoes only a small part is phosphorylated (Reestman *et al.*, 1943). In animal tissues, on the contrary, nearly all the thiamine is present as cocarboxylase (Ochoa and Peters, 1938b; Westenbrink and Goudsmit, 1938). Part of the thiamine in milk is present as mono- and as diphosphothiamine, particularly in goat's milk (de Jong, 1942) and as a thiamine-protein complex (Houston and Kon, 1939; Halliday and Deuel, 1941). The thiamine is readily absorbed from the intestines (Stockholm *et al.*, 1941). It is very rapidly phosphorylated to cocarboxylase in the liver (Ochoa and Peters, 1938b) and in other tissues (Westenbrink and Goudsmit, 1938). In blood plasma and in cerebrospinal fluid there is a very small amount of free thiamine (about 1 γ/100 ml.). Most of the thiamine in blood (6–12 γ/100 ml.) is present in the corpuscles in the phosphorylated form, combined with protein (Sinclair, 1938; Goodhart and Sinclair, 1939). In contradistinction to the cocarboxylase, thiamine is readily diffusible (Banga *et al.*, 1939b). If the intake of thiamine is not excessive, most of the vitamin is used up in the metabolic processes in the tissues; in normal intake (1–2 mg. per day in human beings) about 10% is excreted in the urine (Westenbrink and Goudsmit, 1937) and all is excreted as free vitamin (Westenbrink and Goudsmit, 1939). So thiamine is absorbed as the free vitamin, is phosphorylated by nucleated cells, is dephosphorylated in the kidneys, and is excreted as free thiamine.

Borsook *et al.* (1940) studied the course of the thiamine metabolism by the use of a thiamine prepared with radioactive sulfur. They found a rapid interaction of injected thiamine with the thiamine pyrophosphate present in the blood and tissues, and a rapid destruction of the thiamine. This destruction yields neutral sulfur compounds and inorganic sulfate in the urine.

IX. ANTITHIAMINES

As soon as organic chemistry had succeeded in synthesizing the different vitamins, chemists synthesized substances with closely related molecular composition, and tested whether these compounds had the same or similar biological activity as the natural vitamins. It appeared, in general, that such a hetero-vitamin was inactive (see e.g., Stein *et al.*, 1941), or very much less active than the vitamin itself. Often that such a compound had a reverse action, and worked like an antivitamin. In the case of thiamine, a small change in the molecule had not much effect on its physiological activity. Thus, when in the formula of thiamine



Pyrimidine part Thiazole part
Thiamine hydrochloride

the methyl-group in the pyrimidine ring in 2 was replaced by an ethyl group, the activity of the resulting compound was about the same as that of the original thiamine, as estimated by rat experiments. When the methyl group 2 in the pyrimidine-ring was replaced by propyl, the activity was somewhat reduced in pigeon tests. However, when the same methyl group 2 of the pyrimidine ring was replaced by *n*-butyl, the activity of the 2 *n* butyl homologue was reversed; it had an antithiamine effect as was shown by Emerson and Southwick (1945). Buchman *et al.* (1940) observed that a number of substances, having a structure similar to that of thiamine pyrophosphate are able to inhibit the activity of the carboxylase system markedly. The most active inhibitor they studied was the pyrophosphoric ester of the thiamine thiazole moiety. Woolley and White, (1943a, b) studied the activity of the pyridine analogue of thiamine, 2-methyl-4-amino-5-pyrimidylmethyl-(2-methyl-3-hydroxyethyl) pyridinium bromide, which they called pyrithiamine.

The great importance of these antithiamines lies in the fact that they may be used to obtain a pure avitaminosis from one single vitamin. As we do not yet know all nutrients, it is often difficult to obtain a diet deficient only in one vitamin, but adequate in all other respects. To obtain a thiamine-deficient diet, yeast or liver are often autoclaved at pH 5, or treated with sulfite (Williams *et al.*, 1935; Schultz *et al.*, 1941) to destroy the thiamine. But it is difficult to destroy the last traces of the thiamine. On the other hand this treatment may have a deleterious effect on other nutrients.

So we may expect that the antivitamins will prove in future to be very useful in studying the activity of the separate vitamins. Thus Woolley and White (1943b) state that while mice fed a ration free of thiamine develop no characteristic symptoms the same animals, with administration of pyrithiamine, do show many symptoms characteristic of thiamine deficiency.

X. THIAMINASE

Green, R. G. *et al.* (1941, 1942) demonstrated that chastek paralysis, a disease that occurs in foxes on a diet containing 10% or more of fresh, uncooked fish, could be prevented or cured by thiamine. Woolley (1941) and Spitzer *et al.* (1941) found that a heat-labile principle, probably an enzyme from an extract of carp viscera was able to inactivate a thiamine-solution *in vitro*. Krampitz and Woolley (1944) showed the enzyme activity of the fish principle by isolating the split products from thiamine; 4-methyl-5-hydroxyethylthiazole and 2-methyl-4-amino-5-hydroxymethyl-pyrimidine. Sealock and Livermore (1944) realized that this reaction gave rise to hydrogen ions. By using a bicarbonate buffer in a Warburg apparatus they studied the kinetics of the reaction by measuring the CO_2 that was set free by the hydrogen ion. This enzyme, the thiaminase, is present in several species of fishes, mostly in fresh-water fishes (Deutsch *et al.*, 1943), but also in the Atlantic herring and in aquatic animals like the clam (Melnick *et al.*, 1945).

Perhaps the thiaminase may also be used to study the influence of a pure athiaminosis. Smith and Proutt (1944) stated that cats fed a diet consisting exclusively of raw carp or raw herring developed all the signs of the thiamine deficiency characteristic for this species.

XI. PRACTICAL APPLICATIONS OF THE PHYSIOLOGICAL FACTS

1. *Method for the Determination of Thiamine in the Form of Cocarboxylase*

These are the chemical methods for the determination of thiamine: the thiochrom method (Jansen, 1936), the method using an azo coloric reaction (Prebluda and McCollum, 1936, 1939; Platt and Glock, 1943) and the microbiological methods, especially that of Schopfer (1939).

The thiochrome method is more sensitive than that of McCollum and Prebluda, but even with this method extremely sensitive instruments are needed to determine the small quantities present in 1 ml. or in some drops of blood. The method of Schopfer is better adapted to small quantities, but it takes 10–14 days before the results are obtained.

As indicated above, nearly all the thiamine in blood and in animal tissues is present in the form of cocarboxylase. This cocarboxylase can be measured by its activity as a coenzyme. Ochoa and Peters (1938b) were the first to use this method for the quantitative determination of thiamine and cocarboxylase in boiled tissue extracts. Goodhart and Sinclair (1939) applied this method to the determination of cocarboxylase in blood. All the blood cocarboxylase was found within the blood cells, particularly in the nucleated cells.

Westenbrink (1940) worked out a micromethod, which permits the determination of about 0.00005 γ of cocarboxylase and about 0.0005 γ of thiamine separately in a mixture of both compounds.

Westenbrink *et al.* (1943b) used alkali-washed yeast as an adsorbant and at the same time as the apoenzyme for the cocarboxylase preparation from blood. In this way, using the "cartesian diver" technique it was possible to determine the cocarboxylase content of 0.01 ml. of blood (Parvé, 1945).

2. *Biochemical Assay of the Nutritional Status in Terms of Thiamine*

The fact that thiamine has a decisive influence on the metabolism of pyruvic acid makes it probable that in thiamine deficiency the pyruvic acid content of the blood may be increased. Platt (1938) and Platt and Lu (1939) found a large increase of bisulfite-binding substances mainly consisting of pyruvic acid in the blood of beriberi patients. Some hours after the administration of thiamine to the patients the bisulfite-binding substance content of the blood dropped to normal. Therefore, it may be assumed that the increase of pyruvic acid in the blood is an indication of thiamine deficiency. Bueding and Wortis (1940) found a rise in blood pyruvic acid in alcohol addicts with acute peripheral neuropathy. A large intake of carbohydrate and strong muscular effort (Johnson and Edwards, 1937) in a normal subject also led to a marked increase of pyruvic acid in the blood. In an hour or less, however, the pyruvic acid returned to its resting value. In animal experiments, Thompson and Johnson (1935) found that pigeons and rats with symptoms of acute thiamine-deficiency had a high blood pyruvate. De Jong (1936) confirmed these facts; he devised a micro method for determining the pyruvic acid (measured as bisulfite-binding-substances) in a small drop of blood; thus he was able to make several determinations during the course of each experiment. In this way he proved that the symptoms of acute polyneuritis in pigeons developed before the rise of the bisulfite-binding substances. The disappearance of the symptoms after the administration of thiamine preceeded the return of the bisulfite binding substances to normal. This makes it highly improbable that the symptoms of acute polyneuritis in pigeons might be caused by poisoning with pyruvic acid.

Stotz and Bessey (1942) showed that a variety of conditions, such as excitement, muscular exercise, anoxia and fasting, raise the pyruvic and lactic acid levels in blood. Under these conditions, a rather close relation between the two is maintained. However, even in mild thiamine-deficiency there is a great excess of pyruvic acid in the blood and the determination of pyruvic acid, as well as lactic acid can give a strong indication of thiamine deficiency.

3. The Use of a Thiamine Pyrophosphate Enzyme System as an Indicator for Bringing about the BAL

Peters *et al.* (1935, 1946) showed that iodoacetate and also very small concentrations of mustard-gas sulfone ($0.09 \times 10^{-3}\ M$) and of arsenite ($0.1 - 1.2 \times 10^{-3}\ M$) inhibited the pyruvate-oxidase system that was catalysed by cocarboxylase. This was the origin of the highly important work of Peters and his school during the second World War on the antidotes for the war gases mustard gas and lewisite. They used the pyruvate-oxidase system as an *in vitro* test for the activity of these gases and thus they ultimately were able to synthesize the 2:3-dimercaptopropanol (BAL, British Anti-Lewisite) as a powerful antidote against those war gases and also against mercury and other poisonous metals.

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Niacin in Amino Acid Metabolism

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I. INTRODUCTION

Although it is not the purpose of this article to review the history of pellagra, certain salient points need to be mentioned since much of the work concerned with niacin and amino acid metabolism has been influenced at least in part by previous classical observations. Out of the great number of theories advanced as to the etiology of pellagra the following are of chief concern: (a) diets consisting primarily of corn cause the disease, (b) the disease is caused by a toxin produced in corn, particularly spoiled corn, (c) pellagra is the result of an infectious agent, (d) pellagra is the result of a poor diet deficient in quality protein or the pellagra-preventive factor. As a result of the numerous and classical researches of Goldberger, it was clearly established that pellagra was indeed dietary in origin, and with the later observations by many clinicians that both niacin or niacinamide were specific in the cure of the disease it was generally concluded that a deficiency of niacin was indeed the sole factor involved in its etiology.

The development of a microbiological assay method for niacin (Snell and Wright, 1941) and the application of this most useful tool to the analysis of foods and human diets caused certain doubts to creep into the thinking of those concerned with the relationship of corn to the cause of pellagra. It was evident from the analytical results that corn was not particularly deficient in niacin but had in fact considerably more of this vitamin than did milk, which has long been recognized as a valuable dietary adjunct in the treatment of the disease. A study of the niacin content of cereals by Aykroyd and Swaminathan (1940) emphasized the discrepancy between the incidence of pellagra and the niacin content of the diet. They observed that the maize diet consumed by peasant populations in Rumania contributed nearly 15 mg. of niacin per day yet resulted in a substantial incidence of pellagra, whereas little or no pellagra was found in India among people eating a rice diet which would give from 5 to 11 mg. of niacin per day. This was true in spite of the fact that the maize diet contained more animal protein than the rice diet. As the result of such observations, it was concluded that "no theory of causation of pellagra can be regarded as satisfactory which does not explain the maize eaters liability to the disease."

With these factors in mind, work was initiated at the University of Wisconsin laboratories in an attempt to find out why corn diets resulted in an apparent increased dietary requirement for niacin.

II. THE EXPERIMENTAL STRATEGY

Two animal species (i.e., the dog and the rat) were considered for the experimental approach to the solution of the above question. The use of the dog offered the advantage that considerable information had been accumulated concerning the niacin requirement of this animal. The chief difficulty contemplated in experiments employing the dog resided in the fact that since it required niacin even on the usual artificial diet, it might be difficult to demonstrate a significant and quantitative increase in the niacin requirement that could be attributed solely to corn. Certainly any such experiment would be made more valuable if the observations could be extended to another animal. The rat was therefore chosen as the experimental animal of choice, not because it required niacin, but because it did not. At least no serious evidence had been presented that the rat required this vitamin on an otherwise adequate ration. It was reasoned that a diet containing an appreciable amount of corn might so increase the rat's need for niacin that the customary ability of the rat to synthesize this vitamin would be exceeded and at least a partial deficiency ensue.

III. INFLUENCE OF CORN DIETS ON NIACIN REQUIREMENT

1. *Studies with Dogs*

The niacin deficiency syndrome in dogs has been discussed *in extenso* in a recent review by Handler (1948) and need not be considered here.

An evaluation of the niacin content of the blacktongue producing diet employed by many of the early workers made it clear that they were not extremely deficient in niacin, as evaluated by microbiological assay. The characteristic ration employed in such studies contained about 70% yellow corn and 18% casein. If one assumes a fair estimate for the niacin content of whole corn at 20 γ per g., such a diet would contain at least 1.4 mg. %. Based on a daily food consumption of 35 g. of diet per kilogram of body weight, a dog would receive an amount of niacin at least 25% higher than the minimum requirement for the growing dog, as established by Schaefer *et al.* (1942) using a synthetic ration. This would agree with the observation of Handler (1943) who stated "it seems not unlikely that the presence of corn meal *per se* may be an etiological factor in pellagra."

In an attempt to quantitate such observations, Krehl *et al.* (1945a) found that when corn grits replaced 60% of the sucrose in the usual artificial diet (contains 19% casein) about three times as much niacin had to be added to the ration in order to obtain a growth rate comparable to that observed with the control ration without the added corn grits. Furthermore, when the corn grits supplementation was reduced from 60 to 36%, only 50% as much niacin was needed to give the same growth as was previously observed. It must be emphasized that the level of casein in the corn grits diet was not reduced, in fact the total protein content of the diet was increased by the amount contributed by the corn grits. Dogs which consumed a mineralized milk diet that contributed about 0.25 mg. of niacin per kilogram of body weight per day exhibited better growth than comparable animals receiving a corn grit supplemented diet containing about 1.05 mg. of niacin on the same basis. As a result of these and earlier observations, one must conclude that diets which contain an appreciable amount of corn seem to predispose the dog to the development of blacktongue. Or expressed in another way—"increased the amount of dietary niacin needed to prevent the disease."

2. Rat Studies

In an attempt to extend the above observations made with the dog, Krehl *et al.* (1945b) investigated the influence of corn and corn products in similar experiments with rats. When the usual niacin-free synthetic ration containing sucrose and 15% casein was supplemented with corn or corn grits to the extent of 40% of the entire ration, a marked retardation in the growth rate of the growing rat resulted. Although it was realized that the addition of corn reduced the casein content to less than that of the control (i.e., from 15 to 9%), the growth obtained with the supplemented ration was still less than that which could be obtained with a control ration that contained only 9% casein. All of these rations were reinforced by the addition of 0.2% cystine to compensate for the sulfur amino acid deficit at this low level of casein. The effect of

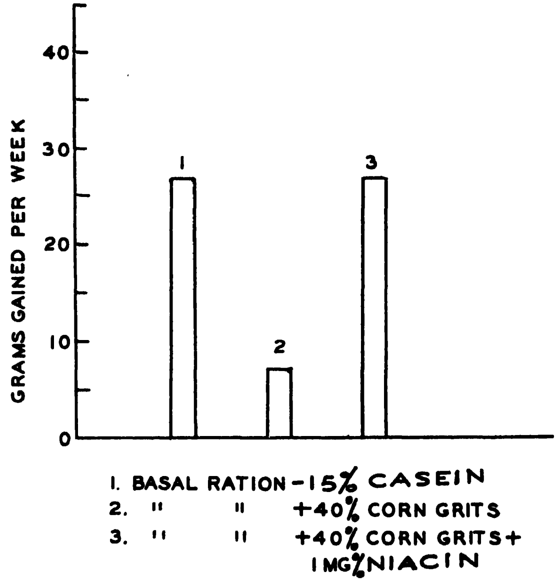

FIG. 1. The deleterious effect of corn grits on the growth rate of rats and the counteraction by niacin.

adding corn grits to the diet on the growth rate is shown graphically in Fig. 1.

Although corn adversely affected the growth rate, it was also reported that the kind of carbohydrate and the level of casein used in the diet modified the extent of growth retardation. When glucose was used in the basal ration instead of sucrose, or when the casein level of the basal diet was increased from 15 to 20% prior to supplementation with 40% corn, growth of the rat was not impaired. It was also evident that corn grits (which consists mostly of endosperm and are devoid of the hull and germ of corn) produced a more severe growth depression than corn itself.

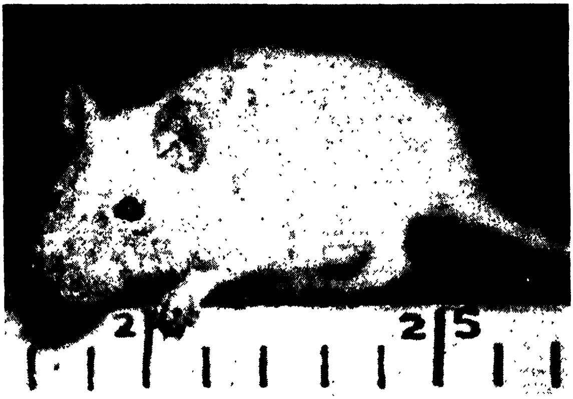

FIG. 2. Appearance of a niacin deficient rat after 4 weeks. Note the unkempt hair coat, porphyrin deposits around the nose, and stunted size.

In addition to the growth depression observed in the young rat given the corn-supplemented ration the animals appeared very unkempt, with a rough fur coat and with occasional alopecia. Also an accumulation of a reddish porphyrin-like material was observed on the paws, nose, and whiskers of the deficient animals. The appearance of such an animal is shown in Fig. 2.

That the corn grits diets had an effect on the rat other than growth retardation was indicated by the fact (Krehl *et al.*, 1946d) that the niacin content of the livers of such animals was significantly lower than the normal and was restored to normal by the feeding of small amounts of niacin.

Levels of niacin ranging from 0.05 to 1.5 mg. % were tested to determine the effective concentration of added dietary niacin needed to counteract the "toxic" action of corn. Although the first level of niacin, which resulted in a significant growth response, was 0.4 mg. %, 1.5 mg. % was required for maximum effectiveness. Niacinamide, ethylnicotinate, *N*-phenylniacinamide, trigonelline, and N^1-methylnicotinamide were tested for growth promoting activity. Only niacin, niacinamide, and ethylnicotinate proved active in promoting growth.

It was also shown (Krehl *et al.*, 1946d) that the corn-supplemented rations had no observable harmful effect on matured male or female adult rats, since such animals could be kept on this diet for as long as 10 weeks and still show the usual gradual increase in weight exhibited by mature rats. The only previous indication that a niacin deficiency might be possible in the rat comes from the work of Frost and Elvehjem (1937) wherein it was observed that niacinamide induced a delayed but definite increase in growth rate. It was also noted that "the addition of adenine nucleotide stimulated growth." A consideration of the ration employed by these workers makes it obvious that they were dealing with a different phenomenon, since their diet contained 18% casein, only 12% corn, and dextrin as the carbohydrate. As will be indicated shortly, a niacin deficiency would not be produced under such circumstances.

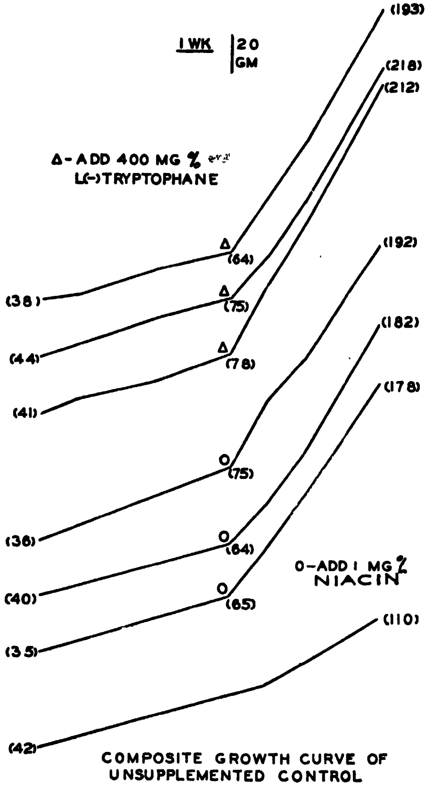

FIG. 3. Growth responses due to the addition of tryptophan or niacin to the corn grits supplemented ration and the growth curve of the unsupplemented control (figures in parentheses represent rat weights). (From Krehl *et al.*, 1945c.)

Since the observations with the rat buttressed those found with the dog, it was evident that an increased requirement for dietary niacin could be created by diets containing large amounts of corn. Although these experiments did not answer the question of why this was so, the work with the rat in particular provided a rapid and reproducible means for a further exploration of the question.

In view of the fact that the above protective action of high levels of casein could not be explained on the basis of its niacin content, Krehl *et al.* (1945c) considered other possible factors. As corn is known to be deficient in the amino acids lysine and tryptophan, it seemed logical that the additional casein (20% level) might be contributing these amino acids in sufficient quantity to overcome the deficiency caused by corn. The addition of lysine did nothing to alleviate the situation, but when either L- or DL-tryptophan was added at levels as low as 50 mg. %, a pronounced growth response was observed (see Fig. 3). This then was a clear cut demonstration of a mechanism involving an interrelationship between an amino acid, tryptophan, and a vitamin, niacin.

An examination of the literature as far back as the work of Lussana and Frua in Italy in 1857 (Harris 1941) reveals the *implied* importance of "an insufficient quantity of dietary protein" in the production of pellagra. Goldberger and Tanner (1924) reported on the value of milk and meat proteins in the treatment of pellagrins. It had also been observed (Goldberger and Tanner, 1922) in several isolated cases that some beneficial results in the treatment of pellagra were obtained from tryptophan and cystine. On the basis of the marked antipellagra action of very small amounts of yeast and because of certain inconsistencies using proteins as therapeutic agents in pellagra, Goldberger and Tanner (1925) reported that "it is possible that the 'P-P' factor plays the sole essential role in the prevention and thus the causation of pellagra."

The above mentioned experiments with the rat show clearly that niacin is capable of playing the "sole essential role," but also show that protein or tryptophan in particular has a pronounced effect on the niacin requirement.

Spector and Mitchell (1946), using the paired feeding technique, confirmed the above observations and demonstrated in the paired-fed groups that "incorporation of niacin or tryptophan in the corn-supplemented ration produced slight but statistically greater gains in the young rats at the same level of food consumption." Both niacin and tryptophan produced a somewhat more efficient use of the ration for growth. Singal *et al.* (1946a) and Dann (1946) also reported confirmation of the original findings.

Although Salmon (1947) confirmed the findings of Krehl *et al.* (1945b) regarding the dual effect of niacin and tryptophan in stimulating rat growth, he could find "no evidence of a specific deleterious effect of corn products in niacin deficient rations." An examination of the reported data reveals, however, that such inadequate growth was obtained on the basal diets used in these experiments that it would be very difficult to demonstrate a further growth impairment on the addition of corn. It is further evident from the data that the niacin contained in the 40% corn meal which was used exerted little growth-promoting activity. This is in accord with the concept that corn does contain niacin, but other factors involved not only prevent complete revelation of its biological activity, but further increases the animal's dietary requirement for this vitamin.

Salmon (1947) advanced the hypothesis of a "niacin-tryptophan-sparing action of fat since when 30% of lard was included in the diet containing 40% corn meal and the casein increased to 13.3% (to maintain the same total protein to non-protein calorie ratio as in the 9% casein, 40% corn meal diet without lard) growth was normal without the addition of niacin." Because of this "fat sparing action," it was indicated that niacin functions primarily in carbohydrate metabolism.

While all of this makes an attractive hypothesis, the author of this review would like to suggest that other explanations are just as attractive and at least as well supported by experimental evidence. It has been shown, for example (Hart *et al.*, 1948), that an increase in the fat content from 4 to 40% results in an enormous rise in the voluntary caloric intake which persists at a high level for as long as two weeks. If such a phenomenon occurred in the experiments mentioned above, it would mean an effective increase in the total casein intake as compared with the controls, since the protein level of the high fat diet was increased to maintain the "same total protein to non-protein calorie ratio."

In a similar vein French *et al.* (1948) observed that a decrease in the protein intake from 22 to 7% of the diet did not alter the superior energy utilization of the high fat diet by the growing rat, and further, that an increased weight gain, increased body gain of fat energy, and decreased heat production were associated with high fat diets. These observations tend to place considerable doubt upon the generally accepted idea that the growing rat will consume equal numbers of calories of "isocaloric low or high fat diets." From a standpoint of human pellagra, little evidence is at hand which would indicate that the fat contributed from the salt pork of the classical pellagra-producing diet of maize, meat, and molasses has any influence on the prevention of the disease.

IV. EFFECT OF DIET COMPOSITION ON THE NIACIN-TRYPTOPHAN REQUIREMENT

1. *Influence of Cereals and Legumes*

In addition to corn, other cereals and legumes, namely, polished rice, whole wheat flour, refined wheat flour, whole rolled oats, peanut flour, cow peas, and soy bean flour, were tested for growth inhibition in a manner comparable to the corn-supplemented diets (Krehl *et al.*, 1946d). Good growth (Table I) was obtained with all cereals other than corn despite the fact that polished rice, rolled oats, and rye all contain less niacin than corn. These results are, in part at least, attributable to the fact that all these materials, with the possible exception of rice, contain enough tryptophan to raise the concentration of this amino acid in the supplemented ration to 150 mg. % or more. Although rice contributes less niacin and very little more tryptophan than corn, the best growth rate of all was obtained with rats fed the rice-supplemented diet. It should be pointed out, and it will be discussed in more detail later, that fair growth could be obtained with rats on niacin-free diets containing only about 0.1% tryptophan, but the addition of corn to such a diet either increased the dietary requirement of tryptophan to about 0.15% or made manifest a niacin deficiency correctable by the addition of 1.5 mg. % of niacin or 50 mg. % of tryptophan. When the dietary level of tryptophan falls below 0.1%, the addition of even large amounts of niacin has no effect. This is, of course, what might be expected since niacin cannot replace the essential amino acid tryptophan as a part of the structural component of tissue protein.

TABLE I

Effect of Corn and other Cereals and Legumes on the Growth Rate of Rats
(Krehl *et al.*, 1946a)

	Total protein	Total niacin	Tryptophan	Gain per week
	%	mg. %	mg. %	g.
Basal C15[1] + corn grits	12.6	0.27	118	7
Basal C15 + corn grits + niacin	12.6	1.27	118	29
Basal C15 + yellow corn	12.4	0.92	129	13
Basal C15 + white corn	12.7	0.91	130	14
Basal C15 + polished rice	12.0	0.56	138	31
Basal C15 + rye flour	14.0	0.41	158	26
Basal C15 + rolled oats	14.0	0.41	168	28
Basal C15 + white flour	14.4	0.40	151	29
Basal C15 + soybean flour	29.0	0.80	348	26

[1] Contains sucrose and 15% casein.

That certain parts of the corn kernel do not inhibit rat growth was made clear (Krehl *et al.*, 1946b) by the fact that corn bran alone or the addition of 5 or 10% corn germ to the corn grits diet resulted in excellent growth.

2. *Effect of Carbohydrate*

a. In Corn Rations. It has been amply demonstrated (Mannering *et al.*, 1944) and (Schweigert *et al.*, 1945) that the type of carbohydrate used in a "synthetic ration" has a marked influence on vitamin synthesis in the intestinal tract. The growth inhibiting effect of corn was tested when fed with sucrose, glucose, dextrin, and lactose as the chief dietary carbohydrates. From the results obtained (Krehl *et al.*, 1946d) it was evident that glucose, dextrin, and to a lesser degree, lactose, exerted a marked influence in correcting the growth inhibition which resulted from feeding corn or corn grits. (The role of intestinal synthesis in this problem will be presented in more detail later.)

When milk was fed at a level of 5 or 10 ml. per day as a supplement to the ration which contained corn, a correction of the growth deficit was obtained. These results are in accord with the classical observations concerning the action of milk in the prevention and treatment of pellagra, and may be due in part to the influence of lactose as indicated above. It hardly seems likely that these small amounts of milk would contribute significant amounts of tryptophan. Neither butter fat nor corn oil at a level of 25% in the diet proved effective in correcting the growth inhibition observed with the corn rations.

b. In Noncorn Rations. Since it was evident that the growth inhibiting effect of corn was related to an apparent deficit of tryptophan in the corn protein, an effort was made (Krehl *et al.*, 1946d) to discover whether other proteins low in tryptophan had a similar effect. With this in mind, diets containing 15% wheat gluten and 10% gelatin were tested, using sucrose, dextrin, glucose, and corn starch, respectively, as the carbohydrate component of the ration. The rats exhibited the same growth inhibition, followed by growth stimulation after the addition of niacin and tryptophan with the above protein mixture, and with sucrose as the carbohydrate as had been observed with corn diets. Thus it was found that "corn is not unique in producing severe growth failure (in the rat) and that the corrective interplay between niacin and tryptophan is not restricted to corn-supplemented rations." It was furthermore noted that dextrin and glucose exerted the same protective action in the wheat gluten-gelatin ration against the deleterious effect of tryptophan deficiency that had been noted in the corn rations. When corn starch was used as the carbohydrate in the above diet, good growth was not obtained until niacin or tryptophan were added.

It appears therefore that the efficacy of carbohydrate in counteracting the niacin deficiency in the rat decreases in the following order: dextrin, glucose, corn starch, and sucrose. At about the same time, Briggs (1945) reported that the observations regarding niacin and tryptophan in the rat could be extended to include the chick and also that "tryptophan may successfully replace niacin in a purified diet not containing corn." The use of 10% gelatin as a source of arginine and lysine in an artificial chick diet caused a marked depression of the growth rate and produced the typical niacin deficiency symptoms (Briggs *et al.*, 1943). The "inhibitory effect was not evident when gelatin was replaced by arginine and glycine, although the growth rate was not quite optimum." It is interesting to note in these studies that 18% casein in the diet of the chick does not provide protection against a niacin-tryptophan deficiency, whereas this amount is ample for the rat. In this sense the chick behaves more like man, dogs, and pigs than does the rat.

3. Character and Amount of Dietary Protein

To further emphasize the importance of amount and character of dietary protein, especially with regard to tryptophan content, Krehl *et al.* (1946c) showed that egg albumin, fibrin, and soybean globulin at dietary levels of either 10 or 15% protected the rat from the usual growth inhibition which resulted when corn grits were added to the diet. This is in marked contrast to the results obtained with casein at comparable levels and is undoubtedly dependent upon the higher tryptophan content of these proteins, as compared with casein.

The specificity of tryptophan in this relationship is indicated at least by the fact that addition of lysine, arginine, methionine, and histidine to the corn supplemented ration at levels of 0.5% failed to elicit a growth response.

That this relationship of level of dietary protein and niacin requirement is important for species other than the rat and chick will be remembered from the work with pigs, reported by Wintrobe *et al.* (1945). On niacin-free diets containing 26% casein young pigs "showed no signs of nutritional deficiency except for a slightly less satisfactory growth in certain instances." At a level of 10% casein, however, omission of dietary niacin "was associated with the development of signs of nutritional deficiency (markedly impaired growth, rough coats, diarrhea, poor appetite and severe anemia)."

The importance of dietary protein and tryptophan in the nutrition of dogs on a niacin deficient diet has been reported by Singal *et al.* (1948a). They noted that niacin deficiency in dogs was "not prevented or significantly altered by the inclusion of the tryptophan-deficient proteins, zein, or gelatin, in the diet." Their basal diet contained 19% casein. This is in contrast to the findings with the rat. While a basal diet supplemented with 21% casein (total casein 40%, tryptophan 0.48%) did not prevent niacin deficiency in the dog, the "basal diet supplemented with as little as 0.1% DL-tryptophan containing a total of 0.28% tryptophan is effective in this respect." This is further evidence (which will be discussed again) that supplementary tryptophan as the free amino acid is metabolized in a different manner than when it is fed as an "integral part" of the protein molecule.

4. Concept of Amino Acid Imbalance

Although previous experiments with wheat gluten and gelatin indicated that an undesirable growth rate might result when niacin and tryptophan are simultaneously low, a niacin-free ration was tested, (Krehl *et al.*, 1946c) containing 9% casein (the amount contained in the earlier corn-supplemented rations) and 6% gelatin. Sucrose, dextrin, glucose, and corn starch, respectively, were used as the carbohydrate component in the diets. In all cases (see Table II) growth inhibition was produced by the addition of gelatin in much the same way as when wheat gluten had been used instead of casein. In all cases the addition of niacin or tryptophan resulted in good growth.

TABLE II

The Growth Inhibiting Effect on Rats of Gelatin and Zein in Diets Containing Different Carbohydrates and the Corrective Action of Niacin and Tryptophan
(Krehl *et al.*, 1946b)

Ration used	Type of carbohydrate used			
	Sucrose	Dextrin	Glucose	Corn starch
	g./wk.	g./wk.	g./wk.	g./wk.
Basal ration	10–13	29	21	26
Basal ration + 6% gelatin	3	10	11	10
Basal ration + 6% gelatin + N[1]	18	24	21	20
Basal ration + 6% gelatin + T[2]	22	23	20	22
Basal ration + 3% zein	3			
Basal ration + 3% zein + N[1]	22			
Basal ration + 3% zein + T[2]	23			

Basal ration—carbohydrate 84%, casein 9%, corn oil 3%, salts IV 4%, plus L-cystine 0.2% and adequate amounts of water and fat soluble vitamins.

[1] Niacin.

[2] Tryptophan.

Since this is the same condition that prevails in corn-supplemented rations, the effect of zein, the principal protein in corn, was tested by adding it at a level of 3% to a 9% casein ration at the expense of carbohydrate. (It will be noted that this level of zein is about that contained in the corn-supplemented rations used before.) This addition of zein resulted in very poor growth (Table II) which was correctable by the addition of tryptophan or niacin.

As a result of such experiments, Krehl *et al.* (1946b) proposed that "the evidence so far obtained indicates that the action of corn in niacin-low synthetic rations is a function of the character of the predominate protein present, zein, and this in turn is due to an amino acid imbalance in this protein." It was further stated "that an imbalance of amino acids results in an increased tryptophan (or niacin) requirement, and therefore poor growth, is evident by the fact that rats will grow reasonably well on rations which contain the same amount of tryptophan and which also contain a well balanced protein."

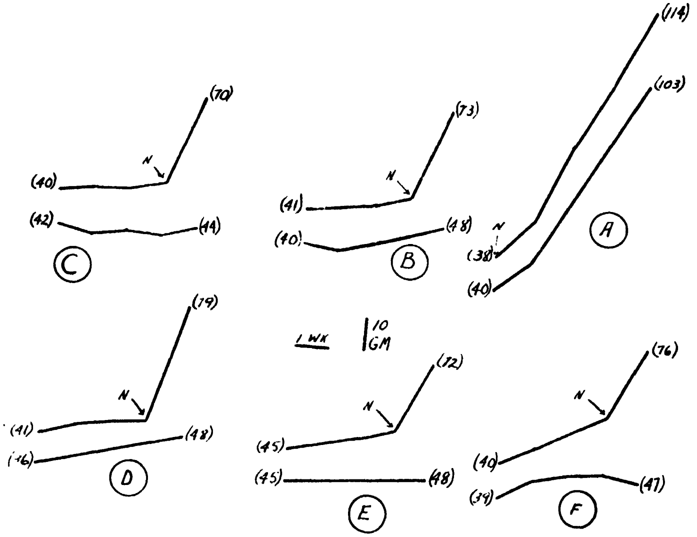

FIG. 4. Growth responses in rats on various diets when supplemented with niacin (N) at the indicated point in the growth curve. Group A, 9% casein (sucrose) basal with and without niacin; Group B, 9% casein (sucrose) basal plus 2% acid-hydrolyzed fibrin; Group C, 9% casein (sucrose) basal plus 2% acid-hydrolyzed egg albumin; Group D, 9% casein (sucrose) basal plus 2% acid-hydrolyzed casein; Group E, 9% casein (sucrose) basal plus 6% gelatin; and Group F, 9% casein (sucrose) basal plus 2% glycine. (Krehl *et al.*, 1946b.)

It was evident at the same time (Krehl *et al.*, 1946b) that this imbalance of amino acids could be extended beyond proteins such as gelatin and zein, when it was noted that the addition of protein hydrolyzates (acid hydrolyzed to destroy tryptophan) such as fibrin, egg albumin, and casein at levels as low as 2% inhibited growth. This inhibition could be counteracted by niacin even after three weeks on the inhibitory regimen. (See Fig. 4.)

5. Influence of Specific Amino Acids

Since such findings indicated the possible involvement of a specific amino acid, glycine, leucine, and glutamic acid were added separately and collectively at a level of 1% to a 9% casein-dextrin ration. No growth inhibition was observed, but when sucrose was used as the carbohydrate in place of dextrin, the addition of 2% glycine and a combination of L-tyrosine, DL-phenylalanine, and DL-valine along with glycine and L-leucine resulted in a retarded growth rate which was markedly stimulated by the addition of niacin.

Groschke and Briggs (1946) also observed that the addition of certain pure amino acids alone and in combination to synthetic diets low in niacin produced growth inhibition in chicks analogous to that noted with gelatin. Niacin in all cases overcame this inhibition. Glycine alone, arginine and glycine together, and "especially arginine, glycine, and alanine in combination showed marked inhibitory action." These authors suggest that niacin "is concerned in some manner with the metabolism of amino acids especially glycine, arginine, and alanine."

In a further study on growing chicks and on laying hens, Briggs *et al.* (1946) report that niacin deficiency may occur in chickens under "practical conditions if the protein (amino acid) content is unbalanced" and "that the niacin requirement of chickens is influenced by the protein content of the ration and in particular by the presence of excessive amounts of proteins such as gelatin which are deficient in tryptophan."

Groschke *et al.* (1947) also pointed out that the "chick-pellagra symptoms" caused by feeding zein are due to the accumulated action of the amino acid constituents alone in this protein. Of the amino acids in zein, glutamic acid, leucine, alanine, proline and phenylalanine seemed primarily concerned.

An indication of nonspecificity of amino acids in this mechanism was indicated by the above mentioned work with rats and chicks and was further pointed out by the report of Groschke *et al.* (1948) when they found that at a level of 4% in the diet, nearly all of 17 amino acids tested depress the growth rate to some extent. DL-Methionine proved particularly effective in this respect.

Another study on the amino acid imbalance affecting the growth of rats on a niacin-tryptophan deficient diet was reported by Henderson *et al.* (1947). Growth retardation was observed in rats when the 9% casein-sucrose, niacin-free diet was supplemented with 2% glycine, 2% acid hydrolyzed casein, or an amount of crystalline amino acids equivalent to 2% acid hydrolyzed casein. Again the growth depressing effect of glycine could not be shown on a diet which contained dextrin as the carbohydrate.

Singal *et al.* (1947) working along a similar line with rats noted that "the addition of histidine, valine, threonine, and lysine, to the low protein (9% casein) diet produces a marked growth depression which is not accompanied by a decrease in liver niacin."

Hankes *et al.* (1948) were able to demonstrate that the specific amino acids which most effectively induced a niacin-tryptophan deficiency in the rat were DL-threonine and DL-phenylalanine in amounts equivalent to that present in 2% acid hydrolyzed casein, i.e., 0.078% and 0.104% respectively. DL-Phenylalanine was not as effective as DL-Threonine and furthermore the growth inhibition with these amino acids was not marked when dextrin was used instead of sucrose as the carbohydrate. This observation is reminiscent of the previous work reported above.

Singal *et al.* (1948b) confirmed the above observation concerning the growth retarding action of added dietary threonine for the rat and further noted that the growth inhibition is not accompanied by a decrease in the storage of niacin in the liver or muscle. It will be recalled that the usual niacin deficiency in rats produced with corn rations results in a decrease in liver niacin.

One must ask the question whether these effects of single amino acids are only a small part of a much more elaborate metabolic scheme. It must certainly be evident that the effects shown with gelatin are not attributable to the very small amounts of threonine and phenylalanine contained in this protein. Observations such as these are perhaps only the initial wedges which may be helpful in prying into the complicated web that guards the secrets of the metabolic relationship between the amino acids and the vitamins.

V. Role of Toxic or Pellagragenic Factors

1. *Historical*

Lombroso, the famous Italian "pellagrologist," was perhaps the earliest and foremost advocate of the so-called "toxic theory," as the cause of pellagra. He suggested that a primary poison in corn was not responsible, but that toxins developed in spoiled corn through the action of certain microorganisms, which were in themselves harmless to man.

In more recent times Chick (1933) rejected the idea that pellagra is actually caused by an amino acid deficiency or to "vitamin B_2" deficiency, since "they did not adequately explain the association of epidemic pellagra with the consumption of maize." Chick proposed the theory that "pellagra is caused by a toxic substance derived from the maize diet which can be corrected by sufficient good protein or perhaps by sufficient vitamin B_2." Of course, as has been pointed out earlier, the remarkable efficacy of niacin or niacinamide in the treatment of human pellagra soon relegated all "toxic theories" to the status of "old wives tales," and replaced this idea with the concept of a vitamin (niacin) deficiency as the real cause of pellagra.

2. *Antimetabolite Concept*

A renewed interest in the possibility of a "toxic" factor in corn which may be conducive to a niacin deficiency stems from the more or less recent observation that compounds can be made or found in nature which are structurally similar to but biologically antagonistic to other important metabolites. It is thought that these structural analogues produce specific signs of deficiency by acting as a competetive inhibitor to the metabolite in question.

Woolley *et al.* (1938) found that dogs suffering from niacin deficiency were "promptly killed" by a single dose of 3-acetylpyridine. To observe whether 3-acetylpyridine was behaving as a structural analogue, Woolley (1945) was able to produce typical signs of niacin deficiency in mice by giving this compound as a single dose of 3 mg. The symptoms of the deficiency were prevented by adequate amounts of niacin or niacinamide in the ration, but little success was reported when a biologically active niacin derivative was used in curative attempts. In a later paper, Woolley (1946a) also reported the reversal of the deficiency produced by 3-acetylpyridine on the addition of as little as 0.1% tryptophan to the diet. It should be noted, however, that the diet used in these studies contained 18% casein, which would supply adequate amounts of tryptophan for niacin synthesis in the rat. Whether the same protein level needed for protection holds for the mouse as for the rat is not clear.

Whereas Woolley and White (1943) reported that pyridine-3-sulfonic acid did not produce niacin deficiency in mice, Krehl *et al.* (1946b) observed that the addition of niacin to a low-protein ration containing this compound did stimulate growth. No growth inhibition could be observed, however, by the inclusion of 3-acetylpyridine in the diet at a level of 0.2%. These results with pyridine-3-sulfonic acid may have been an artifact in view of the latter observation that some strains of rats require more dietary niacin than others at low levels of protein (Krehl *et al.*, 1947).

More specific evidence for the existence of a pellagragenic agent in corn was presented by Woolley (1946b) who found that "a chloroform and NaOH extract of the grain" produced a diarrhea, reddening of the skin and tongue, and a reduced growth rate in mice, all of which was curable with niacinamide. Although some steps were made to purify the causative factor, it has not been identified at the time of this writing.

3. *Role of Indole-3-Acetic Acid*

Since maize is known to be a rich source of indole-3-acetic acid, present largely in the form of a precursor, Kodicek *et al.* (1946) included this compound in a low protein diet and observed that it inhibited growth in 19 of the 32 rats tested. Of 33 rats fed a similar maize meal diet, 29 of 33 showed the growth depression.

In the meantime Krehl *et al.* (1947) found that when the casein level of the niacin-free basal diet was reduced to 9 or 10%, growth of the "Yale" strain rat was about twice that of the Sprague-Dawley animal. The administration of niacin to the Sprague-Dawley animals obliterated this difference, but did not greatly stimulate additional growth in the Yale strain animals. Again, in referring to the role of carbohydrate, when the diets contained dextrin instead of sucrose, little difference in growth was observed between these strains. These results are presented in Table III. Bearing in mind this influence of strain difference on increasing the animals niacin requirement, it was found that indole-3-acetic acid and in addition indole-3-propionic and indole-3-butyric acid did not result in growth inhibition even at relatively large levels.

TABLE III

Growth of Two Strains of Rats on Niacin Deficient Diets and the Influence of Carbohydrate and Niacin on Such Growth

(Krehl, 1947)

Ration	Strain	Gain per week, g.
Sucrose, C9[1]	SD[2]	10
Sucrose, C9 + niacin	SD	23
Dextrin, C9	SD	27
Sucrose, C9	Y[3]	24
Sucrose, C9 + niacin	Y	31
Dextrin, C9	Y	30
Sucrose, C15 + 40% corn grits	Y	15
Sucrose, C15 + 40% corn grits + niacin	Y	35

[1] C9 = Casein 9%, C15 = Casein 15%.
[2] SD = Sprague Dawley strain rat.
[3] Y = Yale or Connecticut Agriculture Experient Station Strain rat.

A further study by Kodicek *et al.* (1947) on this subject also revealed that the erratic response of the rat on low protein niacin-free diets was responsible for the misinterpretation of his original observations concerning indole-3-acetic acid. In addition the suspicion is voiced by Kodicek that microsynthesis may be of special importance in the whole problem.

4. Niacin Content of Corn and Dietary Requirement

Since it has been suggested (Burkholder *et al.*, 1944) that varieties of corn might be developed which would contain larger natural levels of niacin, an experiment was conducted by the author with the collaboration of Professor R. F. Dawson of Columbia University to evaluate the nutritional effects of two strains of corn, one of which contained the normal level of niacin (i.e., about 20 γ/g.) and the other a relatively large amount of niacin (i.e., about 50 γ/g. or nearly as much as the average for whole wheat). The results obtained (Krehl, 1947) showed first, that growth on the diet containing the "low-niacin corn" was lower than that on the niacin-free control diet. Second, that growth on the "high-niacin corn diet" although equal to that of the control was only about one half as good as was seen when niacin as such was added to the control diet in amounts equivalent to that contributed by either the "low niacin" or "high niacin" corn. These data again support our original contention (Krehl *et al.*, 1946d) that while corn does contain niacin, it either does not exert a normal biological activity or the amino acid imbalance created by the zein of corn has too great a growth impairing action to be overcome by the niacin which is contained in this cereal.

In a similar vein, in nutritional studies conducted with the duck, Hegsted (1946) found that the addition of much larger amounts of niacin to the corn diet did not result in growth comparable to that observed with their artificial diet.

The results of Luecke *et al.* (1947), working with the pig, indicated that a high dietary level of corn induced a niacin deficiency even though the percentage of protein was "relatively high." The addition of tryptophan to such diets produced animals that were essentially normal.

The production of "experimental pellagra" by the prolonged feeding of rather substantial doses of adenine has been reported in dogs (Raska, 1947). It seems unlikely, however, that this substance is the pellagragenic factor in corn. As pointed out earlier (Frost and Elvehjem, 1937) adenine produced an immediate but not continuous growth response in the rat. Spies and Stone (1948) report that adenine in doses of 2.5 g. twice a day for a total dose of 32.5 g. did not produce symptoms of pellagra although acute toxicity as the result of this dosage of adenine was observed.

5. *Pellagra from Noncorn Diets*

It must not be assumed from all of this that pellagra cannot be produced on diets which do not contain corn. In a most interesting study, made under trying conditions, Lewis and Musselman (1946), reported the occurrence of pellagra in large numbers of the inhabitants of a Japanese prison camp who subsisted on a diet markedly low in calories, B-complex vitamins, and protein, particularly protein of animal origin. The disease in these patients was complicated by other deficiencies such as nutritional edema and beri-beri. Marked improvement was evident in these patients after the administration of B-complex vitamins containing niacin or when the animal protein content of the diet was increased.

Although Briggs *et al.* (1945) reported failure to produce pellagra in human subjects after as long as 42 weeks on a diet containing only a small amount of corn products and contributing only about 4 mg. of niacin per day, it must be pointed out that the patients received some meat, unenriched wheat flour, and oatmeal, all of which seem to be better sources of tryptophan than a diet composed largely of corn.

It is interesting that the per capita consumption of corn in Mexico approximates 280 g. per day. This corn is consumed largely as "tortillas" and forms a very important part of the diet. Despite this, the incidence of pellagra in Mexico is not great. Is it possible that methods used in preparing the tortillas destroy any pellagragenic factor? It might be mentioned that the traditional drink "Pulque" (a fermentation product) contains niacin, and consumed in reasonable amounts could contribute significant amount of this vitamin.

VI. INTESTINAL SYNTHESIS AND THE NIACIN-TRYPTOPHAN RELATIONSHIP

The many attempts to elucidate the niacin-tryptophan relationship have centered around two main ideas: (a) that bacteria in the gastrointestinal tract synthesize the vitamin and (b) that niacin synthesis from tryptophan takes place in the animal's tissues. Since hindsight offers a better point of vantage than foresight, it seems evident now from the variety of experiments at hand that both types of synthesis may occur and play important roles in niacin metabolism.

The early observations concerning the marked influence of carbohydrates in promoting growth in rats receiving corn diets prompted Krehl *et al.* (1946d) to state that "the effect of different carbohydrates on the growth depression might be explained on the grounds that they contribute to the establishment of an intestinal flora which is capable of synthesizing adequate amounts of the deficient factor." While it is recognized that technical difficulties hinder the unequivocal demonstration that the animal's need for niacin can be synthesized by bacteria and adsorbed from the intestinal tract, enough indirect but nevertheless valid data has been presented to show that such a mechanism may play a significant role, the extent of which is influenzed in part by the animal species in question.

In a study on the intestinal synthesis of niacin and folic acid in the rat, Teply *et al.* (1947) noted that dextrin diets caused the greatest synthesis of niacin as evidenced by analysis of the animal's cecal content.

Hankes *et al.* (1948) observed an inverse relationship between the niacin content of the feces and growth, and suggested that although niacin synthesis in the lower tract may not contribute toward the nutritional requirement of the rat, niacin synthesis in some portion of the tract does.

The role of the intestinal flora as an important source of niacin in the human has received strong support from the extensive studies of Ellinger *et al.* (1944, 1945). The results of these workers indicate that the production and release of niacin by the intestinal flora can amount to as much as 80% of "the daily uptake." They suggested that this may explain inconsistencies observed in the etiology of pellagra. Concerning the previous mentioned effects of milk in counteracting niacin deficiency, these authors point out that milk favors the production of a more elaborate intestinal flora and one favorable for niacinamide synthesis through the use of the amino acids of milk proteins which were used as the building blocks in this synthesis. It will be noted that lactose also has a favorable effect on the establishment of an intestinal flora capable of contributing to niacin synthesis.

Najjar *et al.* (1946) have also produced evidence that the synthesis of niacinamide by bacteria in the intestinal tract is an important factor in contributing to the human requirement for this vitamin.

In a later report Ellinger (1947a) found that when fecal coli were reduced as the result of feeding succinylsulfathiazole, the customary increase in N^1-methylnicotinamide excretion following oral doses of tryptophan was reduced "to $\frac{1}{5}$ or $\frac{1}{2}$ of the original increase." It was further shown that ornithine can be utilized *in vitro* for niacin synthesis by coli and that arginine and glutamine which have NH_2 groups in the delta position and which presumably can be converted into ornithine have a similar effect. When a mixed culture from the rat intestine was incubated *in vitro* with tryptophan, an increase in niacin production resulted.

Regarding the biological fate of N^1-methylnicotinamide, Ellinger (1947b) makes the interesting observation that this substance may be eliminated in the bile and destroyed (also synthesized) by intestinal bacteria, excreted in the feces or be reabsorbed from the intestine. Because of these varied fates, it was considered that "faecal output cannot be used to estimate the niacinamide state of the body."

It has also been shown (Ellinger, 1946) that the excretion of N^1-methylnicotinamide is reduced by the addition of corn to the diet, while the addition of milk to the diet increased excretion somewhat.

Other complications in the role of the intestinal flora in niacin synthesis are indicated: (a) by the observation of Koser and Baird (1944) that niacin destruction may occur during the cell multiplication of certain organisms, (b) from the fact that glucose can inhibit growth in a bacterial media devoid of niacin (Kligler *et al.*, 1943), (c) that corn at high levels can reduce the bacterial population in the rumen of sheep (Onderstepoort, 1943), and (d) that with *in vitro* experiments Benesch (1945) showed that under aerobic conditions considerable niacin synthesis occurs in feces obtained by cecostomy while under anaerobic conditions the cecal organisms destroyed about $\frac{2}{3}$ of the niacin present in the original material.

On the basis of the above work it would seem reasonable at least to retain the hypothesis that the microflora of the intestinal tract is capable of profound alteration as the result of dietary changes. And further, that such changes may react to the benefit or detriment of the host, depending upon the extent of vitamin synthesis or destruction that these changes can induce.

It is evident that dietary circumstances which have a known influence on the character of the intestinal flora must be taken into consideration if an adequate appraisal of the nutritional status of an individual is to be made.

No evidence has been produced thus far which refutes the idea that intestinal synthesis may play an important role in the niacin-tryptophan relationship. Until such evidence is produced, this concept must be given consideration.

VII. Concept of a Direct Tryptophan-Niacin Interrelationship

1. *Influence of Niacin on Tryptophan Utilization*

While it is obvious from what has been said that a metabolic relationship exists between niacin and tryptophan, the exact nature of this relationship was not made evident. That the intestinal flora played a role seemed clear, yet such an explanation was not completely adequate. In a further attempt to clarify the relationship between niacin and tryptophan, Krehl *et al.* (1946a) determined the balance between ingested tryptophan and the tryptophan accounted for by gain in body weight plus that excreted in feces and urine in rats on corn grits diets with and without added niacin. Although the tryptophan content of the two rations was identical, i.e., 118 mg. %, little growth, and also little niacin synthesis was in evidence unless niacin was added to the extent of 1.5 mg. %. From what has been said before about the growth inhibiting action of zein, it would appear that the inability of the rat to grow can be attributed to the amino acid imbalance which results from feeding this protein. On the other hand, it might be explained that the tryptophan content of the corn ration is adequate in the presence of added dietary niacin because niacin exerts its effect by increasing the metabolic utilization of dietary tryptophan for tissue synthesis. In support of this contention, it was found that the addition of niacin increased the "utilization" or accountability of ingested tryptophan from 30 to about 70%.

It was further suggested that "the good growth effected by the addition of tryptophan to corn rations could result from the action of tryptophan in improving niacin synthesis which could then play a dual role of providing adequate niacin for tissue growth and improving tryptophan utilization."

Hundley (1947) in work with rats noted a marked diminution of niacin synthesis in the niacin-tryptophan deficiency syndrome with a resultant deprivation of tissue niacin and depressed growth. The explanation was offered that niacin acts to "spare tryptophan," since in niacin deficiency tryptophan must be diverted from tissue synthesis to niacin synthesis.

2. Urinary Excretion Studies

a. Rat and Other Experimental Animals. Huff and Perlzweig (1942) in a study on the metabolism and synthesis of niacin in the rat observed that "the chief end product of niacin metabolism in the rat is trigonelline as it is in man and in the dog." It is, of course, recognized that the true excretory product, N^1-methylnicotinamide, was converted to trigonelline by the analytical method used at that time. The observation that such a metabolite in urine could be used as an index of niacin metabolism was applied to a study of the effect of numerous sources of nitrogen on the level of N^1-methylnicotinamide excreted in the urine. Choline, DL, 8-amino-*n*-valeric acid and glycine were fed in the diet to rats, and on "the very first day the trigonelline excretion of rats in all three groups was increased" by 74, 149, and 117% respectively. The addition of casein increased "trigonelline" (N^1-methylnicotinamide) excretion in male but not female rats. Interesting enough after ammonium lactate was given intraperitoneally "the increase in the trigonelline excretion in the urine was immediate and much greater than that obtained with the injection of glycine in the preceding experiment."

Huff and Perlzweig (1943) also established the chemical nature of the substance discovered in urine by Najjar and Wood (1940) which is capable of giving a blue-fluorescence after the extraction of alkaline urine with butanol. This substance was identified as the quarternary ammonium ion, N^1-methylpyridinium, 3-carboxylic acid amide, which they designated N^1-methylnicotinamide. It had also been demonstrated by Najjar and Wood (1940) that the concentration of this substance increased in the urine following the ingestion of niacin or niacinamide, With the above experimental evidence as a background, plus the observed ability of tryptophan to replace niacin for the rat on a corn or low protein diet, Rosen *et al.* (1946) made the observation that "the increase in niacinamide excretion on addition of 50 mg. of L-tryptophan when given either orally or parenterally was immediate within 24 hours and much greater than those observed previously in other amino acids," (see Table IV).

TABLE IV

Average Excretion of Niacin Derivatives per Rat per 24 Hours

(Rosen *et al.*, 1946)

Diet	Days	NA[1]	N^1-Me[2]
		γ	γ
15% Casein	1–10	35	90
15% Gelatin	11–18	15	38
15% Gelatin + 50 mg. L-tryptophan, orally	19–22	347	985
15% Gelatin	23–26	43	43
15% Gelatin + 50 mg. DL-tryptophan, subcutaneously	27–28	175	580
15% Casein	29–34	28	35
15% Casein + 100 mg. DL-tryptophan, orally	35–36	325	770

[1] NA includes all niacin derivatives hydrolyzable with 6 *N* HCL by the method used by Huff and Perlzweig.

[2] N^1-Methylnicotinamide as determined by the method of Huff, Perlzweig, and Tilden.

These observations indicated (although this type of evidence is indirect) that tryptophan may be the important precursor of niacin synthesis in the rat. An adequate explanation has not been offered to explain the increased excretion of "trigonelline" following the administration of the other nitrogen compounds in the earlier experiments, which in some respects indicates nonspecificity of response.

Although the increased excretion of niacin derivatives in rats receiving tryptophan was confirmed by numerous workers in other laboratories, Singal *et al.* (1946b) made the additional observation that "niacin or supplementary casein was without effect in this respect although these supplements permitted normal growth when added to the basal diet." In addition to the elevation of the usual niacin derivatives after tryptophan ingestion, the same workers reported an unidentified substance in the urine which is converted to niacin by acid but not alkaline hydrolysis. In a later paper Singal *et al.* (1948b) could obtain no evidence of this unknown urinary substance in the liver of rats fed tryptophan.

In dogs, Singal *et al.* (1948a) have found that the administration of L-tryptophan results in enhanced excretion of this acid hydrolyzable precursor of niacin but results in only a temporary and smaller increase in N^1-methylnicotinamide.

To my knowledge this substance remains unidentified although a new urinary niacin derivative, the 6-pyridone of niacinamide, has been identified by Knox and Grossman (1946).*

The interesting difference concerning the greater effectiveness of tryptophan as the pure amino acid rather than tryptophan as represented in the intact protein, casein, in increasing the excretion of niacin has also been reported by Bell *et al.* (1948). As yet no rational explanation of this phenomenon is available.

Luecke *et al.* (1948) observed that one of the symptoms of niacin deficiency in the pig is low urinary excretion of N^1-methylnicotinamide, which is elevated after the administration of tryptophan. The addition of niacin to a low protein corn ration increased the utilization of dietary tryptophan, since the animals grew at a normal rate on an average of 0.8 g. of tryptophan while without added dietary niacin the daily utilization of 1.8 g. of tryptophan gave only a slightly better growth rate, and as a matter of fact, resulted in a lower efficiency of feed utilization. This observation then would be in accordance with the previously mentioned findings with the rat.

The effects of feeding tryptophan and niacin on the urinary excretion of niacin derivatives on two additional species of animals, namely the horse and the cotton rat, have been reported by Schweigert *et al.* (1947). An interesting species variation was noted in that the cotton rat, which requires a dietary source of niacin, excretes considerable quantities of N^1-methylnicotinamide when tryptophan is fed, whereas the horse excretes more niacin, but little N^1-methylnicotinamide.

b. Excretion Studies in Humans. Both Roberts and Najjar (1944) and Ellinger *et al.* (1945) had reported a decreased N^1-methylnicotinamide excretion in pellagra. It appears difficult, however, to evaluate the practical usefulness of this test as a real diagnostic aid in the clinical evaluation of pellagra.

Sarett and Goldsmith (1947) reported a study with adult human subjects in which they found a lower N^1-methylnicotinamide excretion in persons on a "corn diet" than those on a "whole wheat diet," although approximately equal amounts of niacin were contained in the two diets (see Table V). They also observed that the administration of tryptophan increased N^1-methylnicotinamide excretion and conclude that tryptophan is a precursor of niacin and may well explain the antipellagragenic activity of certain "niacin-poor" protein-rich foods, such as milk.

Perlzweig *et al.* (1947) also reported an elevated excretion of N^1-

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* Henderson has recently reported that this acid-hydrolyzable substance in urine is identical with quinolinic acid (Henderson, L. M. 1949. *J. Biol. Chem.* **178,** 1005–6).

methylnicotinamide following ingestion of tryptophan by both infants and adults. They also report the inability of D-tryptophan to be utilized for this purpose.

TABLE V

Daily Excretion of Niacin Derivatives in Adults on Corn and Wheat Diets

(Sarett and Goldsmith, 1947)

Diet	Days	Subject 1			Subject 2		
		N	N^1-Me	Total N^1-Me	N	N^1-Me	Total N^1-Me
		mg.	mg.	mg.	mg.	mg.	mg.
Corn	1–10	0.7	0.6	19.6	0.7	1.1	20.6
Corn + 5 g. DL-tryptophan daily	11–12	0.5	1.5	19.8	0.8	4.0	25.0
	13–14	0.6	5.3	23.5	0.7	8.5	26.5
	15–16	0.5	7.0	27.6	0.7	9.9	29.6
	17–18	0.6	6.6	26.1	0.7	13.3	30.0
Wheat	27–28[1]	0.7	1.4	16.0	0.8	2.5	15.0
Wheat + 5 mg. DL-tryptophan daily	29–30	0.5	2.3	16.7	0.7	6.6	19.6
	31–32	0.7	7.5	21.9	0.7	10.1	26.8
	33–34	0.7	10.7	23.4	0.6	13.5	26.0
	35–36	0.6	11.3	25.1	0.6	13.8	26.1

Niacin (N) was assayed microbiologically (Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. Eng. Chem. Anal. Ed.* **15**, 471, 1943); *N*-methylnicotinamide (*N*Me) fluorometrically; total N^1-methyl derivatives of niacin chemically.

[1] Data are given for days 27 to 28 only, since there was a decline in the excretion of N^1-Me during the first 10 days on the wheat diet.

3. Organisms Not Having a Niacin-Tryptophan Interchangability

Evidence is at hand to show that the niacin-tryptophan interrelationship does not hold for all organisms. Schultz and Rudkin (1948) could not demonstrate any sparing action of tryptophan on the niacinamide requirements of the fly, *Drosophila*. In fact the niacinamide requirement for optimum growth was found in general to increase with the amount of tryptophan supplied. In addition Kidder *et al.* (1949) have reported that niacin could not replace tryptophan or vice versa for the ciliated protazoa *Tetrahymena gelei*, strain W.

4. Influence of Pyridoxine and Other Vitamins

Pyridoxine deficiency so alters the course of tryptophan metabolism in a number of animal species that the normal metabolic derivative, kynurenic acid, is replaced by xanthurenic acid (Lepkovsky and Nielsen, 1942). Since the metabolism of tryptophan can lead to N^1-methylnicotinamide, Rosen *et al.* (1947) studied the effect of a pyridoxine free diet on this relationship. It was shown that pyridoxine deficiency in rats results in a decreased urinary excretion of N^1-methylnicotinamide after doses of tryptophan. Although the restoration of pyridoxine to the diet of the deficient animals resulted in a disappearance of xanthurenic acid in the urine, indicating correction in this observed abnormality in tryptophan metabolism, the metabolic defect which resulted in the decreased excretion of N^1-methylnicotinamide was not rapidly restored, even after the animals ingested adequate amounts of this vitamin. Pyridoxal was likewise ineffective in restoring normal N^1-methylnicotinamide excretion. No increased destruction of N^1-methylnicotinamide resulted in pyridoxine deficiency nor was there any impairment in the mechanism for methylating niacinamide. The authors conclude that "the data indicate that the abnormal formation of xanthurenic acid in pyridoxine deficiency is not responsible for the impaired tryptophan to niacin transformation."

In an almost simultaneous publication Schweigert and Pearson (1947) also noted the decreased conversion of tryptophan to niacin derivatives in the pyridoxine deficient rat and mouse. In a subsequent publication Junqueira and Schweigert (1948a) noted that the ability of pyridoxine deficient rats to again excrete normal amounts of N^1-methylnicotinamide after restoring pyridoxine to the diet depends on the length of time that the rats are kept on the pyridoxine deficiency. Rats held on the deficiency for 41 days regained their ability to excrete a normal level of N^1-methylnicotinamide. It is difficult to evaluate or to reconcile the results from these two laboratories since different diets and presumably different strains of animals were used.

In an extensive set of experiments on the metabolic interrelationship between tryptophan, pyridoxine, and niacin, Spector (1948) fed groups of rats all of the possible combinations of these substances. Forced feeding of a liquid diet deficient in tryptophan, pyridoxine, and niacin was resorted to as a means of controlling food intake and inducing the deficiencies more rapidly. As a result of these studies, it was concluded that pyridoxine had no significant effect on the conversion of tryptophan to niacin and N^1-methylnicotinamide. It was shown, however, that the presence of niacin increased the formation of xanthurenic acid in pyridoxine deficiency. Furthermore, the presence of niacin allowed for a greater conversion of tryptophan to N^1-methylnicotinamide than when it was lacking. Spector suggested that the differences observed might lie in the "difference in the experimental technique," since the diets used by the previous workers contained more than adequate amounts of tryptophan and were fed *ad libitum.*

Although it is not appropriate in this review to discuss the relative merits and faults of *ad libitum*, paired, and forced feeding, it would seem reasonable to suppose that both the dietary restriction of a control animal receiving an otherwise adequate diet or the forced feeding of a deficient animal presents circumstances that are both abnormal and unphysiological.

C. T. Ling *et al.* (1948), in an investigation of the effect of tryptophan and niacin injection on the level of the total pyridine nucleotides in the erythrocytes in normal and pyridoxine deficient rats, found that the intravenous injection of tryptophan resulted in a significant increase in the pyridine neucleotide level in the erythrocytes of normal rats but not in pyridoxine deficient animals. No such differences could be observed, however, after the injection of niacin to similar groups of animals. Their experiments are interpreted as showing that pyridoxine is needed to convert tryptophan into niacin derivatives, but that "pyridoxine derivatives are not involved in the synthesis of pyridine nucleotides from niacin." It is further suggested that the continued low excretion of niacin derivatives in the urine following refeeding of pyridoxine is the result of rapid gains in the body mass and the concomitant increased storage of niacin derivatives and tryptophan in the body.

While Bell *et al.* (1948) confirmed tne observations of Rosen *et al.* (1947) and Schweigert and Pearson (1947) they show in addition that the influence of pyridoxine on the conversion of tryptophan to niacin is more pronounced when tryptophan is administered as the free amino acid than when it is fed by increasing the casein content of the diet. This observation is reminescent of the aforementioned work of Singal *et al.* (1948a).

That the excretion of niacin and N^1-methylnicotinamide by rats is influenced by vitamins other than pyridoxine is indicated by Junqueira and Schweigert (1948b) who observed that a decrease in the urinary excretion of niacin derivatives resulted from the inclusion of succinylsulfathiazole in the ration without also including pteroylglutamic acid. No such decrease was noted, however, when pteroylglutamic acid was included.

The omission of dietary thiamine and riboflavin or the severe restriction of caloric intake also effected a reduced urinary excretion of niacin derivatives in the rat. The exclusion of dietary pantothenic acid on the other hand had no effect.

VIII. MECHANISM OF CONVERSION

The very rapid increase in the urinary excretion of niacin derivatives following either the oral ingestion or intraperitoneal injection of tryptophan can be well accounted for by a mechanism of tissue synthesis.

Such indirect evidence cannot, however, be used as ultimate proof that tryptophan is the actual precursor of niacin anymore than one could state that carvone or chloretone are precursors of ascorbic acid in the rat merely because their ingestion results in a large and immediate excretion of this vitamin in the urine.

1. Neurospora Studies

That a mechanism for the conversion of tryptophan to niacin existed was established first by another and most interesting series of researches employing the bread mold *Neurospora crassa.* The use of this organism in the study of biochemical mutations has been extensively reviewed (Beadle 1945a, b). From the work with *Neurospora* has come the concept that biochemical reactions are under genetic control and that by induced gene mutation the synthesis of vital cellular components such as amino acids and vitamins can be altered. Such a mutation becomes evident when growth of the organism becomes dependent upon an external supply of an amino acid or vitamin, etc.

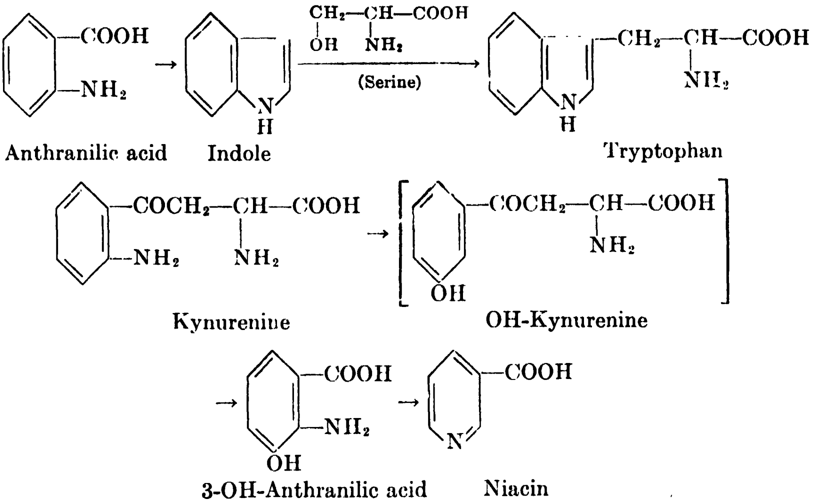
Bonner and Beadle (1946) reported studies with five mutant strains of *Neurospora crassa* which were genetically blocked in the biosynthesis of niacin. Although these workers reported that "no known compound other than niacin or niacinamide has so far been found to possess niacin activity for these mutant strains," when cultured on a medium containing niacin or niacinamide one strain accumulated a substance in the medium possessing niacin activity for one of the other strains. The crystallization of such a substance was reported and its empirical formula established as $C_8H_9NO_3$.

Mutant strains of *Neurospora crassa* requiring exogenous tryptophan for growth were known (Tatum and Bonner, 1944) and the synthesis of indole from anthranilic acid had been demonstrated (Tatum *et al.*, 1944). It was further shown (Tatum and Bonner, 1944) that indole in turn is condensed with the amino acid serine yielding tryptophan directly.

Beadle *et al.* (1947) reported a strain of *Neurospora crassa* which would respond to either tryptophan or niacin, and the behavior of this strain suggested to these workers that it, like the rat, might be able to make niacin from tryptophan. Since kynurenine is a known product of tryptophan metabolism in mammals, this compound was tried as a possible intermediate in the tryptophan to niacin conversion and proved to be active in replacing tryptophan.

On the basis of numerous additional experiments with pyridoxine derivatives, Mitchell and Nyc (1948) concluded that the pyridine ring of niacin is not derived from kynurenine through kynurenic acid or xanthurenic acid followed by oxidation of the benzene ring. These workers then considered the possibility that kynurenine may be oxidized to hydroxykynurenine which would be capable of yielding yet another intermediate, 3-OH-anthranilic acid. When this latter compound was synthesized, and tested as a precursor of niacin, it was found to be active. Bonner (1948) at the same time identified the precursor he had previously crystallized as this same compound, 3-OH-anthranilic acid.

On the basis of these and previous studies, Mitchell and Nyc (1948) proposed the following series of reactions as existing for *Neurospora:*



Bonner (1948) while confirming the tryptophan to niacin conversion and the role of kynurenine and 3-OH-anthranilic acid in this conversion indicated that certain discrepancies exist which are not adequately explained by the above scheme. "Mutant strains are known which accumulate anthranilic acid yet which give no growth response to niacin and there are also mutant strains which can use tryptophane, indole, anthranilic acid, or kynurenine which cannot use 3-OH-anthranilic or niacin for growth. Using appropriate genetic stocks it has been impossible to detect the conversion of tryptophan or kynurenine to 3-OH-anthranilic acid."

2. *Animal Experimentation with Tryptophan Intermediates*

Certainly none of the reactions outlined above, prior to tryptophan, are pertinent for the rat, since anthranilic acid, indole, and indole plus serine had been shown to be incapable of replacing tryptophan or niacin in the niacin deficient rat (Krehl *et al.*, 1946b).

That 3-OH-anthranilic acid can serve as a precursor for niacin in the rat as well as *Neurospora* was demonstrated by Mitchell *et al.* (1948). It is interesting to note, however, that the amount of this compound needed is quite large and in this respect it is more comparable in activity to tryptophan than to niacin. No significant growth response was observed using 10 mg. % of 3-OH-anthranilic acid in the diet, whereas comparable growth responses were observed with 2 mg. % of niacin, 100 mg. % of tryptophan and 100 mg. % of 3-OH-anthranilic acid. These workers reported that 3-OH-anthranilic acid was considerably less active when given subcutaneously.

It has also been shown (Albert *et al.*, 1948) that the excretion of N^1-methylnicotinamide is higher when rats are given 3-OH-anthranilic acid in the diet. It is interesting to note in this connection that rats which had shown about the same growth rate and which received 2 mg. % of niacin excreted almost comparable amounts of N^1-methylnicotinamide as animals receiving 100 mg. % of 3-OH-anthranilic acid. Although it is difficult to place a quantitative evaluation on these data, one might expect under the circumstances a considerably larger excretion of N^1-methylnicotinamide on the diet containing 50 times as much 3-OH-anthranilic acid.

It had been reported earlier (Rosen *et al.*, 1947) that "the administration of kynurenine, kynurenic acid, and xanthurenic acid to rats on a complete diet failed to produce increased amounts of N^1-methylnicotinamide in the urine." It was concluded, therefore, that "the metabolic course from tryptophan to niacin in the rat is not via the kynurenine pathway."

In an attempt to verify the role of kynurenine, kynurenic acid, anthranilic acid, and 3-OH-anthranilic acid, in the conversion of tryptophan to niacin, Krehl and Bonner (1948) tested these compounds in niacin deficient rats by injecting them intraperitoneally in daily injections for a period of 5 days. It was found that the daily injection of 10 mg. of 3-OH-anthranilic acid had the same activity as 10 mg. of L-tryptophan and 1 mg. of niacinamide. Both L and DL-kynurenine, however, proved inactive at the 10 mg. level for a dosage period of 5 days in two separate experiments. The use of anthranilic acid in the diet at a level of 100 mg. % appeared to enhance the deficiency symptoms to a slight degree. No satisfactory explanation is at hand which explains the apparent inactivity of kynurenine in the niacin deficient rat in these experiments.

Certainly if kynurenine is an active niacin precursor as is indicated in the *Neurospora* work, it does not in any sense compare with tryptophan or 3-OH-anthranilic acid under the experimental conditions employed. It is possible that it is metabolized or destroyed so rapidly that its biological role cannot be fully determined. The final answer to this incongruity must await further experimentation.

Another line of evidence confirming the conversion of tryptophan to niacin is seen in the observation (Schweigert *et al.*, 1948) that the injection of tryptophan into the fertile hen egg results in a small but significant conversion of tryptophan to niacin by the developing embryo. No deductions concerning mechanism of conversion can be drawn from such experiments however.

3. *Isotope Studies*

It is evident that the ultimate proof confirming a tryptophan to niacin conversion and the elucidation of the mechanism of this conversion must be obtained through the use of isotopes. Some evidence of this kind is now available.

Hundley and Bond (1948) fed rats large amounts of carboxy labeled niacin and found that the excreted N^1-methylnicotinamide contained 95.7% of the original concentration of C^{13}. This, of course, is proof of the N-methylation of niacin. Under the conditions of this experiment little if any N^1-methylnicotinamide came from sources other than niacin and body stores. It would seem therefore that in the presence of niacin in considerable excess of the diety requirement, there is little niacin synthesis from tryptophan or other sources. This, of course, cannot be interpreted as an indication that such a conversion does not exist.

The work of Heidelberger *et al.* (1948b) was the first reported in which trytophan containing a tagged atom was fed to experimental animals and the tryptophan metabolites isolated and tested for radioactivity. These workers synthesized DL-tryptophan, beta C^{14}, and fed it to rabbits, dogs, and rats. Kynurenine, kynurenic acid, and N^1-methylnicotinamide respectively were isolated from the urine. Whereas the position of the labelled carbon was reported for kynurenine and kynurenic acid, it could not be demonstrated in the N^1-methylnicotinamide which would have been the case had this compound arisen from the oxidation of kynurenic acid. That such a reaction is not likely to occur had been shown before in studies with *Neurospora*.

Although this work confirmed the conversion of tryptophan to kynurenine in the intact animal, the labeled carbon was lost and it was, therefore, necessary to establish its further conversion to niacin.

For this study (Heidelberger *et al.*, 1948a), DL-tryptophan-3-C^{14} was synthesized and fed to rats, after which the N^1-methylnicotinamide was isolated with the aid of "carrier N^1-methylnicotinamide." Isotopic carbon was identified in the carboxy group of the isolated N^1-methylnicotinamide. Although kynurenine and 3-OH-anthranilic acid were not isolated in this experiment, the authors note as a significant observation "that the carbon atom which is a precursor to the carboxyl group of

3-OH-anthranilic acid becomes the carboxyl carbon of niacin." Although such a study presents conclusive evidence for the conversion of tryptophan to niacin, the exact reaction steps in this mechanism, especially between kynurenine and niacin may not be as clear as has been indicated by the reaction diagrams, since additional studies with *Neurospora* indicate the presence of still unidentified metabolites in this kynurenine to niacin relationship (Bonner, 1948).

IX. SUMMARY

It has been stated that "the cardinal principle in the treatment of all disease is to find the cause and remove it."

A great cycle of experimentation has taken place in the search for the cause of pellagra. The concept of dietary origin gave flower to the classification of pellagra as one of the "deficiency diseases" and since the addition of niacin cured the disease, ergo, a deficiency of niacin must cause it. The haunting refrain of the "zeist" persisted, however, in the statement that "no theory of causation can be regarded as satisfactory which does not explain the maize-eaters liability to the disease."

Studies with the rat, chick, pig, etc., have now given credence to the above idea with experimental evidence that at least in part satisfies this long sought for query about the "toxic qualities of corn."

The use of large amounts of corn in a low-protein, niacin-free diet does indeed provoke a niacin deficiency, and what is more this can be done in a readily available experimental animal, the rat.

The link of tryptophan has been forged into the long chain of evidence which Goldberger and others used in their earlier studies which demonstrated the beneficial effect of quality protein in the prevention of pellagra; all of which was set aside in favor of the newer and more attractive "vitamin deficiency hypothesis."

What is perhaps more important, the basic flaw in the dietary which induces the niacin deficiency has been exposed and related to the nature of the tryptophan-poor protein in the diet and to the amino acids present in such proteins. Thus the concept of amino acid imbalance as a decisive factor in determining the dietary requirement for at least one vitamin, niacin, has been conceived.

The effect of dietary carbohydrate and the resulting role of intestinal microorganisms in contributing to the vitamin economy of the animal plays an important part in the niacin-tryptophan relationship. This serves to re-emphasize the need for evaluating nutritional requirements of an animal not from a simple quantitative analysis of the dietary components alone, but in actual biological feeding trials in which case the interplay which may exist between dietary constituents may be made known.

The evidence at hand indicating the conversion in the tissues of one essential dietary constituent, tryptophan, to another, niacin, serves as a basis for the search for other similar relationships which must exist.

The contributions made by studies with *Neurospora* in the elucidation of the mechanism of conversion from tryptophan to niacin exemplifies the importance in biological research of the use of all the available and more expedient tools in unveiling nature's more obscure secrets. Again it must be noted that the landmarks in nutritional research have been accomplished most advantageously through studies with a variety of species of organisms. Such a method has always been useful for purposes of correlation.

We have yet to understand the role of the elusive pellagragenic agent in corn and further, the mechanism by which an amino acid imbalance induces a niacin deficiency remains unknown.

As always is the case, the advancement of knowledge through experimentation adds to the picture of progress, yet leaves the experimenter with new horizons upon which to focus.

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Diet and Aging

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I. INTRODUCTION

The United States now has over ten million citizens who are more than sixty-five years old. The purpose of this summary is to review recent advances that have a bearing upon the nutritional status of this group.

Any consideration of the nutrition of man during the later years of life must recognize that many interrelated factors create special problems in the nutrition of the aged. Part of these factors concern sociological and economic conditions; part are the result of changing mental outlook as the years pass; and part are reflections of pathological conditions that accumulate with the passage of time. Thus an anemia during the later years may be the reflection of too little income to purchase foods rich in iron such as eggs and meat. It may be the result of restricted food habits so that these products are excluded from the diet. It may be due to physiological changes such as poor digestion from some condition such as achlorhydria.

Some of the factors that tend to create malnutrition during the later years of life are the following: low incomes, poor facilities for food preparation, ignorance of nutritive values, mental deterioration, worry from insecurity, poor cooking facilities, living alone, physical handicaps and lack of exercise. Many special physical factors also contribute. The two most important are poor teeth and lack of hydrochloric acid in the stomach.

The later years of life represent the accumulation of all preceding years in the treatment of the body. The poet has expressed this better than the scientist when he says:

> The moment we begin to live,
> Then we begin to die,
> Into the world we weeping come,
> Our whole life tells us why.

Modern research with animals indicates how profoundly both the life span and the terminal diseases of old age are modified by such factors as growth rate during the earlier periods of life. Furthermore, inheritance has a tremendous influence on the length of life and conditions of old age. Throughout life such physical influences as exercise and the amount of excess fat carried by the body influence the nutrition during the last fraction of the life span.

II. Animal Selection for Research in Gerontology

In the 13th century the monk, Roger Bacon, discussed the possibility of research in gerontology using various animal species of short life span. Bacon noted the great advantage of man due to his relatively long life. Until modern times, however, only limited progress could be made because too little was known about nutrition. In most cases animals would have died from nutritional deficiencies unless maintained on natural feeds.

Animal species from insects to man can be used for research. Insects are useful for certain types of nutritional studies involving growth rates, and levels of ingestion of substances such as vitamins and essential amino acids. Research with insects is rapid and inexpensive. Furthermore, complications from symbiotic relationships can be eliminated in some species such as the common cockroach, *Blatella germanica,* by the use of germ free methods. In addition to the cockroach, the common wheat weevil, *Tribolium confusum,* is very useful since its nutrition has been well defined.

Since there are a thousand species of cockroaches and forty-three of these have been recognized in this country north of the Mexican line, there is no dearth of material. Furthermore, cockroaches are omnivorous in food habits although their nutritional requirements for essential amino acids and water soluble vitamins seem as complex as those of higher mammals. The species *Blattella germanica* is distinguished from higher species in needing no vitamin A and carries through all its life-functions without this factor (Bowers and McCay, 1940).

Among the smaller mammals the most useful are mice, rats, and golden hamsters. All three species pass through a span of life terminating in senility at the end of about two years. Among these three species only the pathology of the rat has been studied carefully at the end of the life span (Saxton, 1945). The great defect of the white rat for the study of old age is that it acquires a chronic lung disease about middle life. This is such a dominant terminal disease that this species does not live long enough to develop the common cardiovascular troubles of aging man. However, the rat is very useful for numerous studies in aging.

Mice are also advantageous for many studies such as those dealing with tumors. The extensive background in the genetics of mice makes them especially useful. Their disadvantages are small size, diseases similar to rats, and a tendency to scatter feedstuffs making balance studies almost impossible.

The most useful animal for research on old age is probably the dog. The diseases of this species in old age are very similar to those of man. These include the usual tumors, afflictions of the kidneys, the cardiovascular system, the gastrointestinal organs, and even calculus deposits on the teeth. Moreover, the nutritional requirements of the dog are very similar to those of man except that the dog does not get scurvy (McCay, 1946b).

Another great advantage of the dog is that this species has the greatest variability among breeds, with a widespread difference in size. Even the internal metabolism varies as is well recognized in the case of the uric acid metabolism of the Dalmatian.

The background knowledge in regard to the genetics of dogs is extensive. Researchers can work with pure breeds due to the registration system in common use.

The dog is large enough for adequate sampling of such materials as blood, urine and feces. Procedures for accurate physiological measurements such as those of blood pressure are well established in contrast to similar measurements upon the rat.

There are about a million registered dogs in America so the available material is extensive. One drawback is the long lifespan of the dog, which may exceed twenty years. This makes it necessary to study different dogs during various periods of the life span rather than to study the same animals through the total span. The chief disadvantage of research with old dogs is the expense.

Since old dogs suffer from many of the diseases of old people, they must have constant and careful veterinary attention. Even their teeth must be cared for or they may lose them and be unable to eat.

The housing of old dogs is expensive because kennels must be heated and a high degree of sanitation maintained. Facilities for exercise, sunshine and healthful environment must be provided. Many of these factors are commonly neglected in research with dogs and such studies yield questionable results.

Even though older dogs can be obtained as gifts from kennels, the cost of maintenance runs about two hundred and fifty dollars per year, per animal.

In spite of these disadvantages, the dog is probably the best investment for research in gerontology.

Other species of animals can be used but usually present special difficulties. Large numbers of old sheep go to market each year when about seven years old. This material has been little studied. Such sheep often have defective teeth. Herbivorous species also afford very difficult problems in feeding synthetic diets (Madsen *et al.*, 1935).

Guinea pigs have been little used for research upon aging. Minot (1908) made profitable use of this species, as well as rabbits, for his early growth studies. The guinea pig is promising, also, because it can be reared using germ-free methods (Reyniers, 1943).

Possibly some of the many species of pocket mice may prove valuable. These have been captured and maintained for pets from time to time for

TABLE I

Age at Death in Days of Individual Syrian Hamsters

Males	Females	Males	Females
160	137	675	485
168	214	781	489
234	246	896	504
308	316	942	538
371	329	1200	556
418	405	1200	594
447	420		638
483	424		662
484	432		710
505	442		805
505	442		1012
537	443		1300
582	444		1300
674	479		

periods of five or more years. They can be kept upon mixtures of seeds and need no water in addition to that derived from food.

The Syrian or golden hamster may also prove useful in the study of aging. A group of 27 female and 20 male hamsters were kept in our laboratory until they died in order to get some data upon the span of life. They were fed a commercial dog feed of the type described elsewhere (McCay, 1946b). This was supplemented with sprouted oats.

The span of life of this species is similar to that for the white rat, but probably somewhat longer. The mean and median values are typical for small groups of rats. The extremes are substantially greater. The values are probably low since our laboratory has had limited experience in feeding and caring for this species.

A later experiment using ten of each sex of this species gave mean values of 541 days for females and 674 for males. In contrast to the rat and man the male of this species seems to have a longer mean span of life than the female. This may not prove true when data from larger groups are available.

III. INHERITANCE AND SPAN OF LIFE

In the human species the importance of "selecting proper parents if one desires a long life," has been well recognized at least since the time of Alexander Graham Bell.

Little evidence is available concerning the relationship of inheritance to the life span of animals. The basic data for the study of this problem may be available for race horses and registered dogs but no one has made use of these records.

Morris *et al.* (1933) found that rats could be selected and fed on the basis of efficiency of the conversion of feedstuffs into body tissues. Dr. Kennedy ran one study of the life span of these rats comparing those of high and low efficiency in food utilization. In private correspondence she reported:

> "The average span of life for the high male is 521 days and for the high female, 690 days, for the low male, 500 days, and for the low female, 647 days. I don't know that the difference is significant or not but the data is in line with other facts we have found out about the two strains. The low strain has higher basal metabolism and a higher respiration rate which might tend to show that this rat lives faster than the high strain rat.
>
> "Another interesting fact is that in old age the low strain rat has a strong tendency to produce tumors. This is especially true in low strain females. Many of these growths are mammary, but some are

> internal. The male also has these growths. I do not know whether they are of a malignant nature or not. Some of these rats also develop a bright red eye in old age. The eye looks like a sac of blood instead of the normal black bead."

In one study at Cornell (Sperling *et al.*, 1946) the effect of inheritance could be partly estimated. In the production of large numbers of weanling rats for life span studies our laboratory follows the practice of exposing one female to several males in order to insure a maximum production of offspring. Records are kept on cards indicating the dam of each litter but the sire is unknown.

At the end of a life span study involving six large groups which had been established on the basis of distribution by litter mate and sex, consideration was given to the relation between litter of origin and total span of life. Thus it was discovered that a small fraction of the dams contributed a substantial per cent of the long-lived rats while another small fraction of the mother rats had provided a large number of short-lived rats.

These data indicate the profound importance of inheritance and of litter mate distributions in conducting life span studies in relation to diet or environmental variables. A genetic approach to this problem might also produce more uniform animals than are available today for research in gerontology.

One of the great needs today is for the discovery of a small species comparable to the rat that suffers from cardiovascular disease in old age.

IV. Mineral Metabolism in Old Age

Although some men ingest approximately five times as much sodium chloride during each year of life as do others, no study has ever been made of the effect of varying levels of common salt upon the span of life. During recent years interest in salt metabolism has been revived due to the extensive use of rice and fruit juice diets to relieve hypertension (Kempner, 1946). This diet is very deficient in many factors such as fat soluble vitamins, calcium, and salt. In some cases it contributes further to the suffering of the patient by the attacks of the fruit juices upon the teeth, although this can be avoided by mixing rhubarb juice with the fruit juices to provide one part per thousand of oxalic acid. The merits of this rice-fruit juice treatment for hypertension are still under debate. Current research in this field will undoubtedly contribute to further knowledge of the optimum level of sodium chloride in the diet during the later years.

Better insight into the salt metabolism during all ages has been afforded by two modern texts (Gamble, 1947; March, 1946). Specific consideration has not been given to aging in either one.

V. CALCIUM

Although the crust of the earth abounds in calcium, this element affords one of the major problems in human nutrition. Most of the problem is easily solved in theory because of the wide distribution of limestone, but is difficult in practice because men have not learned to introduce this material adequately into their foodstuffs either in youth or old age. Two examples illustrate this problem. While going over the nutritional status of natives of Okinawa (McCay, 1946a) it was found that the estimated calcium ingested per person per day was less than a fifth of that accepted as a standard requirement in America. In other words, the ingestion was less than 200 mg. daily. At the same time these natives were living above huge caves with walls of calcium carbonate through the underlying strata of coral rock. Since the water supplies came from these caves some calcium was obtained from drinking water and absorption on vegetables during cookery, but not an amount that is considered essential.

Likewise, in many areas where the population derives a substantial fraction of its food from some source such as cod fish, one finds the essential calcium of the head and bones of the fish commonly thrown back into the ocean. Such natives need to learn to modify their cookery, possibly by the use of a food acid such as vinegar, to render the head and bones of fish an acceptable food.

For a century the need of calcium in the development of sound bones for the young, growing animal has been stressed. Although the seriousness of broken bones in older people has long been known, only recently have we recognized the importance of calcium in the diet of the aged. The meager literature in this field has been reviewed by us (Kane and McCay, 1947). New research with old rats and hamsters was also described.

Old animals between 500 and 600 days of age were compared with young growing ones in their ability to absorb dietary calcium incorporated in a complete diet at levels of 0.3, 0.5, and 0.7%. Young animals could store 78% to 88% of the calcium from such diets during growth but old ones could not even maintain their body stores at the lower level of calcium. Such old animals are comparable to men over fifty years of age. Later unpublished studies indicate that this tendency to go into negative calcium balance starts about the beginning of the last third of life in the rat.

This study was later extended to include a senile Newfoundland dog, aged 12 years. During consecutive periods the calcium in the diet of this dog was raised from 0.3 to 0.7% of the dry feed. Although the daily losses of calcium from the body of this dog was only one-fifth as great on the high, as on the lowest level, the dog was always in negative balance. Unfortunately this study was terminated by an accidental breaking of the femur which refused to knit and the dog died before higher levels of calcium could be fed. No one knows how long this dog had been losing calcium from its bones nor how much this fatal accident was a reflection in old age of faulty nutrition during preceding years.

Unpublished data with old rats indicate that other dietary factors that have little influence during early life affect the absorption of calcium. Thus high fat levels in the diet of the old rat can throw the animal into negative calcium balance if the dietary ingestion is marginal, such as a 0.3% level. High melting fats cause more losses in the form of soaps than low melting ones.

Furthermore, the ability of old rats to select diets adequate in calcium seems to decline as age advances (McCay and Eaton, 1947). In early life the young rat has the ability to select a diet containing calcium. In middle life this ability declines but persists for this element while it is lost for others such as protein. In old age the rat becomes an "old fool" and will die while drinking a 10% sugar solution to satisfy its taste rather than eating a diet to meet its nutritional requirements. Thus in old age when the body's need for calcium in the diet increases, the ability to select this diet seems to disappear.

Balance studies with a group of older women (Roberts *et al.*, 1948) indicate that a gram of calcium per day is needed for maintenance. The women used in this study ranged from 52 to 74 years of age. These studies indicated that women on low calorie diets ranging from 1337 to 2089 calories per day could ingest sufficient calcium to remain in balance. One vegetarian who consumed little milk, cheese, or eggs, ingested 846 mg. of calcium from her self-selected diet but did not decrease her losses when supplements of Ca and P were added.

The subject who naturally ate a diet rich in starch and sugar, due partly to living alone, was losing calcium regularly each day in an amount that exceeded 100 mg. The one who derived her calcium from the limited amount found in baked goods was also in negative balance on her self-selected diet. The woman who was subject to the most emotional strain tended to lose essentials during periods of disturbance and regain them between these periods. Each of these individuals was typical of thousands of people in our population and serve to introduce the basic problems of improving the practical diets of aged people.

In the survey of 18 women living in almshouses, Pyke *et al.* (1947)

estimated the daily calcium intake at 0.4 to 0.7 g. with values for calories ranging from 1079 to 1863. In contrast they found the daily calcium ingestion by one group of old men living in a large institution ranged from 0.9 to 1.1 g.

Even middle-aged men may be in negative calcium balance for long periods. Thus Clarke (1926) found his group of San Quentin prisoners stored calcium during the 28 weeks of the balance study. This was undoubtedly the reflection of a long preceding deficiency of both the element and other factors such as vitamin D.

In considering the entire life span of the rat, Sherman and Campbell (1935a) concluded that increasing the calcium of a diet already considered adequate, improved both growth rate and life span. However, Sperling *et al.* (1946) found no increase in life span of rats fed a diet typical of northeastern United States, but "improved" by the addition of more milk and other foods to provide more calcium and vitamins. Likewise, Shields and Mitchell (1946) found no effect from feeding different levels of calcium during the entire lives or rats. In one case their rats were reared upon a diet containing 0.6% and then maintained upon 0.22% until death. Their groups were small, however, and the variability great. Hence the life span of the latter group might have been shortened but the significance not found because of the small numbers involved. Moreover, since the major cause of death in old rats is due to failure of the lungs, this organ and its infections may destroy the animal long before its bones suffer too severely from depletion.

The use of calcium balance to evaluate the need for this element in old age represents only one of many approaches to the problem. Failure to discuss the numerous studies indicating the importance of the endocrines in maintaining the calcium of the bones does not mean that the importance of these factors is not recognized. They fall outside the scope of this summary.

The importance and intricate mechanism of the calcium deposition in soft tissues has been stressed recently in the review by Lansing (1947). The diet undoubtedly influences the calcification of soft tissues. However, this influence is probably due to factors other than calcium. In the course of two decades of research with rats we have seen groups at the time of death with heavily calcified arteries and kidneys while parallel groups were relatively free. This calcification of soft tissues was due to unknown variables in the diet and could never be related to dietary calcium.

This result seems logical inasmuch as the bones represent a reservoir that holds more than 98% of the body calcium. Until these bones are depleted in this element to such an extent that life would probably be impossible it is likely that they can provide the blood and soft tissues with the relatively small amounts of calcium needed for pathological changes. Hence it cannot be expected that dietary calcium would have much specific effect upon the calcification of soft tissues.

Little is known about the problem of the absorption of calcium in the aged. Among individuals this absorption may be affected by the hydrochloric acid secreted into the stomach. Achlorhydria increases regularly with age in man. Ivy (1942) in reviewing data in this field indicates that achlorhydria is found in about every fourth person over the age of 60. No one, however, has run calcium balances and determined the occurrence of achlorhydria upon the same group of people. However, it is well recognized that calcium absorption is much depressed in young animals lacking hydrochloric acid in the stomach.

The form of calcium in the diet of the aged may be far more important than it is during the first third of life when the body seems able to make excellent use of such diverse forms as the sulfate and phosphate.

If absorption from the gut is the primary failure in old age, an old animal should excrete radioactive Ca^{45} much more rapidly in the feces than if the loss of calcium is primarily due to increased excretion into the gut. This technique is now being applied in our laboratory in studies with old dogs and rats but progress is insufficient to be certain of results.

During wartime our surveys indicated that the calcium content of the average naval diet was higher than estimated, probably due to low estimated values for bread and neglect of calcium adsorbed from water in the cooking of vegetables (McCay *et al.*, 1945). However, these surveys only covered the diet of an active group of young men fed upon the choice foods of the nation and upon a very liberal ration.

The picture of the daily nutrition of the aged is quite different. In the course of observations in the mental hospitals of New York State during 1947, it was observed that the mean bread consumption was about 50% higher than that in the Navy. At the same time the cash food allowance was about half as much and the daily calory consumption about 2000, or less than two thirds of that for a typical man in the Navy. This bread consumption indicates the heavy reliance upon this item for food in institutions with populations of high age and low food allowances. Furthermore, bread eating is increased due to poor dentures.

This brief introduction stresses the importance of improving the bread for the aged in order to include more calcium and protein in the dietary. This improvement can be made by including 6 to 8% of dry skim milk and 6% of soy flour into the bread formula. On a dry basis this bread contains 0.11% Ca and 0.21% P. This improvement has now been made in the dietary of the 105,000 patients in the mental hospitals of the State of New York.

The changes in teeth of animals in relation to aging have long been observed but given little attention in experimental science. The older work of Huidekoper (1891) can still be consulted with profit since it reviews the changes with age of various species including swine and dogs.

The teeth of rats subjected to varying rates of growth and varying in age from 474 to 1369 days of age have been examined by Smith (Smith and McCay, 1947). In contrast to the slowing down of deterioration in organs of rats retarded in growth, the teeth seemed to decay more rapidly at an earlier age. The decay of teeth seemed to follow a cycle of two peaks with the latter of these in old age when it became quite marked.

The poor condition of teeth must be one of the dominant factors in modifying the food habits of the older population of America. With more than two million people attempting to subsist on the national average of thirty-eight dollars allowed for old age assistance during the year 1947, there could be little margin for dental services. Both the low income and the condition of the teeth force a change toward a cereal diet, often consisting largely of low quality bread. In the field of gastrointestinal physiology no one can evaluate the effects of regular "gumming" of food followed by swallowing in large chunks. This, combined with a lack of gastric juice containing acid, may well lead to special problems in nutritional physiology.

Smith (1946) has noted the erosions of teeth during periods of mental disturbance. He ascribes this to modifications in composition of the saliva. This may be a factor of some importance due to the increase of insecurity in the aged.

The erosion of the teeth from acid beverages such as lemon juice may prove of increasing importance to the aged due to the use of citrus juice in attempts to cure arthritis and constipation. The teeth of these people may have been etched at an earlier age by the extensive use of a highly acid beverage such as the ubiquitous cola drinks (Stafne and Lovestedt, 1947).

Since the acid erosion of teeth by such beverages as lemon juice can be prevented by the inclusion of one part per thousand of oxalic acid, some natural source of this acid such as rhubarb juice may find use in treating the constipation of the aged (McCay, 1947b). A mixture of equal parts of rhubarb juice with lemon juice or with lemonade will protect teeth entirely from erosion. Rhubarb has long been used as a laxative.

Since the diet of the average older person is already subnormal in its content of calcium, an ion such as oxalate that ties up calcium may represent an unfavorable factor. Recent unpublished studies in our laboratory at Cornell indicate that this wastage can be much reduced by spacing the ingestion of oxalate and calcium. The rhubarb may be consumed at breakfast and the milk at noon or later. By still further spacing the ingestion it may prove possible to minimize calcium wastage.

The question has been raised concerning the deleterious effects of daily ingestion of small amounts of oxalate. Individual dogs were each fed .5 g. of sodium oxalate daily for a period of 15 months without any detectable toxicity and without any evidence of calculi in the kidneys or bladder as determined by x-ray photographs at the time of posting.

Since Hammarsten (1936) was able to produce small calculi by feeding very large amounts of spinach to rats there is some possibility that there may be species differences. Her levels were proportionately much higher than those fed to dogs and those liable to be consumed by older people. Since many common foods such as cocoa contain moderate amounts of oxalate and since the diets of the aged are marginal in calcium, this subject of oxalate metabolism warrants further study.

VI. OTHER INORGANIC ELEMENTS

Modern science has often raised questions concerning other elements but provided no answers. The earlier literature concerning such substances as magnesium has been reviewed by us previously (Cowdry, 1942).

One question of importance that is difficult to answer concerns the use of iodized salt during the later years. The lowering of basal metabolism in man during old age is an accepted phenomenon. Whether or not the raising of this metabolism by thyroxine would prove favorable to older people is unknown. Furthermore, this problem is not readily attacked by the use of older animals since no one has proved that basal metabolism of rats and dogs declines with age (Will and McCay, 1943). Moreover, in the rat, studies of the effects of drugs during the second year of life are really reflections of the given drug upon the progress of bronchiectasis since this is such a prevalent affliction during this period.

VII. DIETARY FAT IN LATER YEARS

The fondness of older people for fat during the later years of life has been the subject of frequent comment (Vischer, 1947). However, Meyer (1947) believes the aged eat too little fat. The gradual loss of fat deposits from the skin and the body's attempt to compensate for this has been given as one reason for this taste, but no concrete evidence supports this theory.

The well-fed older men in the study of Pyke *et al.* (1947) ate more than 90 g. of fat daily in 8 cases out of 12, although the calories ranged only from 2050 to 2251 daily.

In most institutions the allowance of fats for spreading on bread is modest because of the cost. This tends to lower the amount of fat ingested especially when meat, milk, and eggs are limited. In such institutions, however, there is often extensive wastage of edible fats which are trimmed from meats and made into soap. In a program to conserve edible fats for human consumption in the mental hospitals of New York State, it was estimated that a monthly saving of 20 tons of fat was effected when an active salvage program was introduced into half the hospitals. This saving allowed each person an increase of an equivalent of 12 g. of fat daily.

Tests to compare the effect of butter and margarine upon the life span of white rats have been reported by von Euler *et al.* (1947). These workers made certain that their diets were adequate in vitamins A and E. No differences in length of life, terminal diseases or reproductivity were found. Among their four experimental groups the age at death of the oldest rat varied from 702 to 766 days. The terminal diseases were the familiar ones of pneumonia and nephritis with kidney damage including calcareous deposits.

In the studies of von Euler *et al.* (1947) one group of rats was given an extra supplement of 500 γ of "vitamin E." This had no detectable effect upon the mean age of the group. This lack of effect was probably due to the adequate level of vitamin A in the diets. Hence the additional vitamin E did not make more essential vitamin A available to the body.

Earlier studies in the Cornell Laboratory indicated that vitamin E might be so low in diets that the fertility of the male was lost, but still the mean span of life was not affected (McCay *et al.*, 1943). In this study evidence was presented that males could become sterile early in life, as judged by loss of sperm motility, but the total span of life was normal.

The supplementing of a diet containing 5% cottonseed oil with 0.1 mg. of mixed tocopherols per 100 g. of body weight of middle-aged rats, did not increase the total span of life if the vitamin A level were high (Paul and Paul, 1946).

VIII. VITAMIN A AND LONGEVITY

Since the earlier studies of Sherman and MacLeod (1925), the premature deaths of rats fed diets low in vitamin A has been recognized. In this early work comparison was made between a small group of rats fed dry skim milk and dry whole milk as one third of the diet. The other two thirds consisted of whole ground wheat. The rats on the full-fat milk had a mean span of life of 746 days while the other group lived an average of only 369 days. This higher value of 746 days is typical for rats fed diets considered adequate but is only about half the potential span of life for a rat. This latter value exceeds 1400 days (McCay *et al.*, 1935).

Varying levels of vitamin A were fed by Paul and Paul (1946) to small groups of rats throughout the life span. The levels used daily per 100 g. of live weight were 1, 2, 4, and 20 international units. The mean span of life for rats fed the highest level was 649 days. This was significantly higher than the mean of 521 days for rats fed the 4 unit level. The growth rates in each case reflected the level of vitamin fed. They concluded that the optimum level of vitamin A was about 10 U.S.P. units of vitamin A per 100 g. of body weight. This is about five times the minimum requirement.

In a human study the plasma vitamin A and carotene were determined in a group of 155 older individuals ranging in age from 40 to 90 days (Kirk and Chieffi, 1948). No differences were found in comparison with a younger group ranging in age from 16 to 39 years. "The average concentrations of vitamin A, total carotenes, and α and β-carotenes in plasma were 17–23, 200–320, and 100–190 γ %, respectively."

IX. Water Soluble Vitamins

Few attempts have been made to determine the effects of varying levels of B vitamins fed during the whole span of life to animals or during the later years to man. If poverty forces the aged to eat more carbohydrates the need for thiamine will rise. Stephenson *et al.* (1941) found some improvement in part of the members of a group of older people to whom they fed supplements of dried yeast, thiamine, riboflavin, niacin acid and ascorbic acid. Meyer (1947) has reviewed the more recent evidence for the need of additional vitamins by older people. The need seems evident but is difficult to establish.

Attempts were made a decade ago (Drummond *et al.*, 1938) to determine the effect of suboptimal amounts of the vitamin B complex upon the life span of rats. The chief variable in their diets was the replacement of 15% of wheat flour by an equal amount of wheat germ in the better diet. A number of minor variables were introduced. The rats on the better diet lived longer in two laboratories where the work was carried on at the same time. In one laboratory, however, the poorer rats were superior to the better in the other, as judged by the criterion of life span. This was never explained.

Recent attempts were made to compare the typical diet consumed in the northeastern part of the United States with this same diet "improved" by the inclusion of liver to replace half the muscle meat, by doubling the milk, by the substitution of whole wheat for white bread, and by the replacement in the typical diet of part of the potatoes and sugar by natural sources of vitamins provided by tomatoes, carrots, and alfalfa.

The last item was used due to the difficulty of getting supplies of fresh vegetables during wartime.

These "improvements" were futile as far as extending the life span of rats could serve as a criterion (Sperling *et al.*, 1946).

In this same study another group of 80 rats was used to test the "improvement" resulting from the addition of synthetic vitamins to the basal diet of eggs, 3; whole dry milk, 4; margarine, 2; butter, 2; hamburger, 10; pork sausage, 4; white bread (not enriched), 16; potatoes, 31.7; tomatoes (canned) 3; navy beans, 1; carrots (fresh), 7; apples, 10; and salt, 0.3. The following supplement was given each rat weekly: vitamin A, 840 I.U., thiamine, 300 γ, pyridoxin 300 γ, riboflavin 20 γ, pantothenic acid 550 γ, nicotinic acid 4 mg., and filtrate factor 54 units. This supplement also failed to increase the life span above that found for rats fed the basal diet alone. These results cast substantial doubt upon improvements liable to be effected by programs for the "enrichment" of bread with water soluble vitamins. However, such data do not evaluate results that might be anticipated for man in optimum performance in living.

In studies with rats from weaning until old age, Mills (1948) has found only one of the water soluble vitamins was needed in greater amounts during old age than during growth. This was thiamine.

From estimated values, Pyke *et al.* (1947) concluded the diets of the older age groups studied by them were adequate in vitamins except for ascorbic acid. In some cases this vitamin was eaten at a level of only 6 mg. daily. In the case of one individual this value was only 2. The highest recorded among the five groups studied was 43 mg., which is substantially lower than the value of 75 mg. commonly recommended in this country.

X. PROTEIN IN OLD AGE

Nitrogen balance studies (Kountz *et al.*, 1947) upon 27 elderly patients indicated that 40% were in negative balance. A daily allowance of 1.0–1.2 g. per kg. of body weight proved insufficient. In some cases even 2 g. per kg. per day were inadequate. The authors believed these negative balances reflected poor food habits, incomplete absorption, and metabolic changes in the aged.

In contrast to the conclusions of Kountz are those of Roberts *et al.* (1948). Nitrogen equilibrium was maintained on an average intake of 57 g. of protein daily. The variation among individuals was from 34 to 94 g. daily. Although one woman seemed to require 1.3 g. of protein per kg. daily, the other seven maintained equilibrium upon 0.7 to 1.0 g. per kg. of body weight.

From calculated values and summaries of earlier nitrogen balance studies, Pyke *et al.* (1947) set the need for protein by old women at a level of 42 g. daily. About a fifth of the women studied, living at home and in almshouses, did not receive this amount. In contrast the men all exceeded an estimated need of 54 g. daily.

The quality of protein becomes important during the later years of life due to the general tendency to change to food from plant sources. Few studies have considered this trend. Carlson and Hoelzel (1947) found omnivorous rats outlived those fed a vegetarian diet. Since their diets consisted of natural foodstuffs it is difficult to attribute differences to the protein alone.

Black found a high basal metabolism in old rats was associated with a high protein diet in comparison with a low one (1939). The basal metabolism of man is known to decrease in old age but this has not been found to happen in the case of old rats or old dogs (Cowdry, 1942). Since the low basal metabolism of old age in man may be a protective mechanism, the possible elevation either by high protein diets or thyroxine may be unfavorable during the later years of life.

XI. Bulk Formers in the Diet

In no period of life is the proper functioning of the intestine more essential than in the later years. Classical writers among the ancients stressed the importance of the pleasures of relief. In old age many of life's pleasures depart, but those of relief remain.

Little attention has ever been paid by nutritionists to the bulk formers of the diet in spite of their importance in the functioning of the intestine. Fifteen years ago attempts were made to determine the effect of feeding two diets containing respectively 10 and 20% of ground cellulose upon the span of life of rats (McCay, Ku, *et al.*, 1934). The first diet consisted of casein, 25; salt mixture, 4; lard, 20; sugar, 36; cellulose, 10; vitavose (wheat germ concentrate), 5; and cod liver oil, 3. The second diet was similar except that it contained 26% sugar and 20% cellulose. For comparison, a stock diet, long used for production of rats, was used.

The mean lengths of life for the three groups of male rats were the following:

Stock diet (75 male rats)	503 ± 12 days
Stock diet containing 10% cellulose (8 male rats)	674 ± 35 days
Stock diet containing 20% cellulose (9 male rats)	602 ± 61 days

These exploratory studies indicate that the life span was not prematurely shortened in spite of the high levels of cellulose in the form of ground cellophane. Further studies with this material indicated that it irritated the intestine. In spite of this irritation the favorable effect of adequate bulk must have offset the injury.

Hoelzel and associates have long been aware of the importance of bulk in the diet and also recognized the basic differences in materials that provide this roughage. Recently thay have completed some life span studies with rats (Carlson and Hoelzel, 1948b). Their data can be summarized as follows:

TABLE II

Mean Span of Life in Days of Rats Fed Various Diets

Number of rats used in parentheses (Carlson and Hoelzel, 1948b).

	Males	Females
	days	days
Basal (two thirds dried meat)	638 ± 90 (14)	786 ± 196 (12)
5% Bulk	795 ± 192 (8)	873 ± 116 (7)
10% Cellulose	782 ± 156 (8)	626 ± 119 (7)
Vegetarian-meat	813 ± 223 (4)	836 ± 91 (5)
Vegetarian-meat	538 ± 38 (2)	482 ± 146 (2)

In spite of the great variability of their data, the trend would seem favorable for the inclusion of bulk in the diet.

The differences between herbivorous and carnivorous species in regard to the need for cellulose in the diet is well recognized. Even among rat species there also seems to be unlikeness (Howell *et al.*, 1948). Growing cotton rats die with their stomachs completely filled with hair balls if fed certain types of diets upon which ordinary albino rats thrive. When these cotton rats are fed the same diet containing 10% cellulose, they respond to the diet like the albino rats.

Little is known about the value of different forms of bulk at any period of life. Furthermore, the limited knowledge concerning the fate and effects of such substances as various forms of cellulose and pectin, in the intestine, leaves a vast, unexplored gap in nutrition.

XII. VEGETARIAN DIETS

The complexity of possible deficiencies from vegetarian diets renders interpretations from animal research very difficult. At the same time the problem is important because of the substantial fraction of the world's population that practices vegetarianism.

In 1912 Slonaker used a small group of 16 rats to compare the life time effects of vegetarian diets with omnivorous ones. He found much shorter spans of life for rats fed vegetarian diets.

Carlson and Hoelzel (1947) allowed self-selection of vegetarian diets by rats. Only 25% of the young from such diets survived and the growth of these young was poor, Chinese scholars found that rats fed vegetarian diets lived about the same length of time as omnivorous rats (Chang *et al.*, 1941).

	Vegetarian		Omivorous	
	Male	Female	Male	Female
Life span (days)	540	611	527	527

Carlson and Hoelzel (1947) compared the effects of omnivorous and herbivorous diets upon the life span of rats. For an omnivorous diet they fed a mixture of dried veal, 61.5; veal bone meal, 3; corn starch, 31; dried brewer's yeast, 2; salt mixture, 1.5; and cod liver oil, 1. Their vegetarian diet was the following mixture: whole wheat flour, 50; peanut flour, 10; wheat gluten, 7; lima bean flour, 7; linseed meal, 7; corn gluten meal, 5; alfalfa leaf meal, 5; dried brewer's yeast, 5; and NaCl, 2. Both groups were fed all the fresh lettuce they desired.

The life spans of their rats in days were the following:

	Vegetarian		Omnivorous	
	Male	Female	Male	Female
Number	21	25	19	26
Days	631 ± 187	650 ± 204	659 ± 128	733 ± 112

These data indicate a favorable influence of the diet rich in meat.

All studies thus far represent a bare beginning in the evaluation of diets composed of plant foods. Large numbers of rats must ultimately be used for such research in order to appraise not only the diet but such environmental factors as climate. Such research is essential in order to produce optimum health in the rapidly increasing world population. Increased population and concentration in various geographical areas force human beings to subsist more and more upon primary foodstuffs since they cannot afford both a dense population and the luxury of conversion of plant foods into animal products for human foodstuffs. In times past the slow growth of populations in various areas permitted the evolution of food mixtures suited to agricultural production and maintenance of a population in a restricted region. Today men cannot await the slow evolution of diets based upon human experience. Hence research with small animals that pass rapidly through the span of life must be used to afford criteria of optimum mixtures for human consumption.

XIII. LOW CALORIE DIETS AND LIFE SPAN

A substantial fraction of the population of the world is unable to secure enough food to permit more than modest maintenance. Usually diets obtainable by such peoples fail not only to provide sufficient calories to fatten the body but such diets are usually too low in essential factors such as protein, vitamins, and calcium.

Life insurance companies have long recognized that underweight is a favorable factor during the latter half of life in promoting a long span.

The feeding of low calorie diets to man or animals during the early period of life is reflected in slow growth. During the past two centuries certain biologists have stressed the interrelationship between rate of growth and span of life (Finot, 1909). These biologists have considered the problem in relation to various species rather than among individuals within a species. They have noted that animal species that mature slowly usually live long.

In modern times some attention has been given to interrelationships between growth rates of individuals and length of life. This affords a means of experimental attack upon the terminal diseases of old age since any influence that extends the span of life usually does so by modifying the disease that terminates life.

Much confusion exists in regard to the method of study of growth rate and life span. It seems obvious that if slow-growing animals are separated from rapid-growing ones that the group developing slowly would include sick animals predestined for early death. Since this group of diseased animals can seldom be distinguished from healthy ones that grow slowly, any statistical attempt to evaluate the effects of slow growth under these conditions is usually foiled since the diseased animals that die early after a period of slow growth nullify any favorable effect in terms of life span of healthy animals that grow slowly. If such a group is fed a poor diet they may also be condemned to slow growth and premature death. These explain the results of such experiments as those reported by Sherman and Campbell (1935b). Their studies do not evaluate the effects of slow growth but indicate the effects of nutritional deficiencies or disease during early life in shortening the span.

During the past twenty-one years experiments have been completed at Cornell to determine the effect of slow growth rates upon life span. Retarded growth in experimental animals has been produced by diets deficient in calories but more than adequate in essential nutrients. In such studies the effects of slow growth due to unknown causes is equalized by selecting littermates for experimental groups. Fish, rats, and dogs have been used in such studies but life span data are available only for rats. These data have been reviewed recently (McCay, 1947a).

The technique of using retarded growth provides a method of producing groups of rats that exceed one thousand days of age. Every fourth rat attains this age, whereas under usual conditions few rats ever attain the age of a thousand days. Such rats afford material for the study of extreme age.

The fact that such rats can be produced by control of the diet opens the problem of the extreme age that can be obtained by any given species. This indicates that the accepted extremes may be much too short.

The use of restricted calorie allowances may have a profound effect upon degenerative diseases, and possibly even on those of bacterial origin. Moreschi, working in Ehrlich's laboratory (Moreschi, 1909), found that tumors were very difficult to transplant in otherwise susceptible mice, if the food intake of these mice was restricted severely. Even with the tumors growing well, the mice survive longer on restricted amounts of food.

From the time of our earlier reports (McCay and Crowell, 1934) concerning the increase in the life span of rats, it has been evident that retardation of growth by restricted feeding of high quality diets produces its effect by delaying the onset of terminal diseases including tumor growths, degenerated kidneys, and infected lungs. The detailed pathological studies of Saxton (1945) have extended our knowledge and indicated further that slow growth by these methods delays the onset of degenerative changes. The basic reason for this delay remains a mystery.

In discussing the problem of the lower incidence of certain diseases among rats subjected to underfeeding, Jackson (1937) stated: "On the contrary, the lung disorder appeared rather more conspicuous in the control rats. Similarly middle ear infection, with pus evident on removing the tenmen tympani, was noted at autopsy in 26 of the 44 controls (or 59%), but in only 14 of the 38 test rats (or 37%). This difference may be insignificant; but it possibly represents the result of a selective process, the rats more susceptible to infection having been eliminated by death during the experiment."

Jackson did not keep his rats until the end of life but killed them at the end of 60 weeks. Hence his explanation of the slower onset of disease due to retarded growth is not likely, in the light of life span studies.

The interrelationship between the development of tumors and restricted diets has been reviewed by several specialists elsewhere and need not be given further attention here (Morris, 1945; Tannenbaum, 1942; Visscher *et al.*, 1942).

Little attention has been given to the relation between restriction of diet and diseases of bacterial or virus origin although these diseases may be important at any stage of life. Foster and associates (1944) have found that mice are less subject to poliomyelitis virus if maintained on restricted food intakes or on diets deficient in thiamine.

Even in the social life of a community, Crampton (1944) has shown the profound influence of the rate of growth of children. Food is only one of several factors that determines this growth rate.

Few careful studies have ever been made of isolated groups of people long restricted in food intake. One such population, made the subject of limited study, was that on the Island of Okinawa. Before the recent war this island supported a population of more than a third of a million people. The basic foods were rice and sweet potatoes with a limited amount of fish, soybeans, pork, and poultry products. A few vegetables and fruits such as oranges, tomatoes, bananas, and sweet potato tops were eaten (Schwartz, 1945; Smith, 1946).

TABLE III

Age and Weight Distribution of Okinawa Natives

Age group	Female	Male	Weight range (kg.)
0–4	2,311	2,332	7–12
5–9	3,409	3,391	13–19
10–14	3,188	3,009	22–30
15–19	2,660	2,009	34–51
20–24	1,970	334	41–51
25–29	1,718	468	40–50
30–34	1,450	541	40–49
35–39	1,317	530	40–52
40–44	1,276	458	39–47
45–49	1,278	935	39–50
50–54	1,340	983	37–47
55–59	1,062	770	36–47
60–64	817	633	35–45
65–69	626	448	36–44
70–74	387	255	35–45
75–79	208	84	
80–84	69	30	
85–89	14	3	
90–95	2		

During the war Maloney (1945) noted that these people were heavily infested with intestinal parasites, filariasis, tuberculosis, and some leprosy. However, they seldom had asthma, shock reactions, tachycardia, peptic ulcer and arteriosclerosis. The age distribution from one district (Chinen) indicated a substantial fraction of the people survived until a ripe old age in spite of the ravages of war and a social philosophy that assumes little responsibility for citizens outside the family circle (McCay, 1946).

The age distribution and range of body weight for these natives of Okinawa probably reflects the long continued use of a restricted diet.

Although these age groups are probably two years younger than corresponding groups in America due to the custom of counting age from conception, they indicate the survival and body weight. They serve as a crude illustration of a type of study that might prove of substantial value to the field of gerontology. The distribution of the male population in this table is abnormal for the middle-aged groups since they had been removed by the Japanese for labor battalions.

XIV. Diet and Mental Disorders in the Aged

The determinations of the cause of common mental diseases found in America is baffling but very important due to the vast population of aged citizens now cared for in our mental hospitals. Simms (1940) has noted that debility seems of far more concern to the average person than the rising mortality as age advances. Shock has published a scholarly review of the physiological aspects of mental disorders in later life (Shock, 1944).

The decline in the detoxication powers of the body has been determined by Stern and others (Stern *et al.*, 1945). Using the formation of hippuric acid as a criterion, they found evidence that three times as many patients over 60 in contrast to the younger groups, gave symptoms of faulty detoxication. They could not relate this to any special pathology of old age.

XV. Sex Differences in Life Span

Experiments with rats have occasionally indicated sex differences in response to specific diets in relation to span of life. In the past, few workers have been willing to repeat such studies and have been content if differences were significant according to conventional statistical methods. From our experience repetition of experiments may fail to reproduce these differences which indicates the usual statistical approach may not be sound in these life span studies.

In most experiments recorded in the literature the female of a species such as the rat lives longer than the male. This has long been recognized in the case of man. The reason for this difference is unknown.

This sex difference in man becomes especially important in institutional feeding and care since many more women than men are housed in the homes for the aged. Furthermore, since the average woman is several years younger than her husband she is doomed to about eight years of widowhood. These social factors tend to be reflected in the poorer nutrition of aged women than of men (Pyke *et al.*, 1947).

Only a beginning has been made in relating nutrition throughout life to optimum health and performance. Enough has been learned, however, to indicate that the cooperative efforts of the nutritionist, the pathologist, and other specialized workers in the biological sciences may open the way to attacks upon the degenerative diseases that now terminate life. Thus man may come to realize the dream of Metchnikoff and pass into the sleep of death after a natural old age free from disease.

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The Vitamin Requirements of the Growing Rat*

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I. THE ORAL VITAMIN REQUIREMENTS

1. Introduction

In the early days of vitamin research it was necessary for workers interested in problems of nutrition and biological research to use crude concentrates in their animal diets for supplying the unidentified accessory factors, but as one after another of the vitamins was discovered it became possible to incorporate the pure materials in the rations and now all of the B vitamins essential for growth may be given to the rat in known crystalline form. Many workers in the nutritional field have been concerned only with having a surplus of the known vitamins available to the experimental animal and in some cases constituents which were not required have been added to the diet. Because of the excessive divergence encountered when surveying the supplements used in the different laboratories doing animal work, it seemed expedient to review the literature and, if possible, to establish the minimum vitamin levels indispensable for the normal growth and development of the rat under laboratory conditions. In this literature review we have summarized the conclusions reached by various investigators.

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Many experiments have been of only 4 to 6 weeks duration, with great variation in dietary conditions prevailing; some investigations were conducted with depleted animals while in others weanling rats were used; also, strains of rats differed in their nutritional demands and this influenced the results. Consequently, difficulty was encountered in correctly evaluating the demands of a normally developing animal.

It was necessary to limit the review to the requirements for the growing rat receiving a diet of purified casein, fats, carbohydrates, mineral salts, and vitamins; the excellent work which has been done on the vitamin needs during pregnancy and lactation as well as the studies made with rats receiving sulfa drugs or following the removal of certain organs have not been included.

The schematic methods used by different authorities when outlining their dietary regimens were varied and irregular; some workers interpreted their results in terms of daily intake, some used the amount of supplement per 100 g. of ration as a basis, and others reported the requirements per kilogram of body weight. Consequently, the establishment of a uniform structure on which to base equivalents and comparisons necessitated numerous mathematical calculations.

It has been assumed that a rat will eat 10 g. of food per day and that a normal grown rat weighs 300 g. Using these assumptions as a basis, the essential vitamins and amounts needed, as developed in the literature, have been recalculated where necessary to correspond with the requirements per 100 g. of ration.

The quantities of the different vitamins reported as being indispensable varied over a considerable range. Until more accurate values are available it is necessary simply to average the data.

2. Vitamin A

It is standard nutritional practice to add 2% cod liver oil to the ration and this figure is used irrespective of the potency of the oil; thus animals would be supplied 1700 units/100 g. of ration or 4,000/100 g. depending on whether the material was U.S.P. cod liver oil containing 850 units/g. or the medicinal oil containing 2000 units/g. In some laboratories the procedure has been used of administering an oil containing vitamin A by mouth once a week. Selecting 28 different investigators at random it was found that 14 of them added the vitamins A and D to the diet and 14 of them administered the supplements orally. Work in our laboratory has indicated that rats will grow equally well whether vitamin A is given daily or once a week. The oral treatment has an advantage over the inclusion in the diet, namely, the certainty that the animal receives the desired number of units.

TABLE I

Vitamin A Requirements of the Growing Rat

40 Units/100 g. ration	
Units required/100 g. ration	Investigator
> 30	Irving and Richards (1939)
60	Guilbert *et al.* (1940)
> 100 < 250	Lewis *et al.* (1942)
30	Little *et al.* (1943)
24	Callison and Knowles (1945)
60	Rosenberg (1942)

It was found in this laboratory that if vitamin A as cod liver oil was incorporated in the ration the antimony trichloride test showed that most of the vitamin A had deteriorated. However, extra oral supplementation did not increase the growth response and when animals receiving the ration were sacrificed and their livers assayed they showed a good storage of vitamin A. Either antimony trichloride is not suitable for measuring vitamin A in rations that are not newly made or the animals ate enough of the fresh diet to store the vitamin before it deteriorated.

Table I lists the vitamin A requirements which have been reported; these range from 24 units (Callison and Knowles, 1945) to more than 100 units/100 g. of ration (Lewis *et al.*, 1942). Irving and Richards (1939) have concluded that vitamin A requirements increase with the age of the rat. From these figures a tentative requirement of 40 units/100 g. of ration or 4 units per day has been assigned.

We have raised rats on a synthetic ration receiving 4 units of vitamin A per day orally but the rats grew only 2.3 g./day and a redness about the eyelids indicated the supply of vitamin A was low. Based on this initial experiment it would appear that the rat requires slightly more than 4 units per day or 40 units/100 g. of ration.

3. Vitamin D

The rat has been used for many years to test for vitamin D employing diets that contained a high calcium low phosphorus ratio. No reports in the literature could be found in which rickets developed in rats receiving a purified diet containing a complete salt mixture.

To obtain evidence on the importance of vitamin D in a purified diet a group of 40 weanling rats from our breeding colony was fed the following ration: alcohol washed casein, 18%; corn starch, 68%; salt mixture (Jones and Foster, 1942), 4%; corn oil, 10%; the vitamin B factors were added per 100 g. of ration in the following amounts: thiamine, riboflavin, and pyridoxine, 0.8 mg. each; sodium pantothenate, niacin, inositol and PABA, 10 mg. each; and choline chloride, 0.6 g. A vitamin A distillate was given once a week diluted in corn oil so that the weekly dose was 5500 U.S.P. units.

At the end of 30 and again at 60 days two rats were sacrificed and the tibia and femur joints were split and stained with silver nitrate according to the U.S.P. procedure, but no evidence of rickets was noted. The growth curves were also normal. Twenty of the females were mated with males receiving the same diet and 16 litters were cast, 8 of which lived.

When the young were weaned at the 18th day two comparable groups as to age, weight, and sex were formed, one was continued on the purified diet deficient in vitamin D and the second group was given 500 U.S.P. units of irradiated ergosterol once a week as a source of vitamin D.

Within 2 weeks the animals receiving the vitamin D-free diet had scanty coats of fur but this condition spontaneously healed and the two groups of animals were normal in appearance for the duration of the experiment.

The growth rate of the animals receiving the vitamin D deficient diet was slightly less than of those receiving vitamin D, but at the end of the 12th week the weights of the males were comparable; at this time some of the females were pregnant so their weights would not be representative.

The most striking difference occurred when the second generation animals were mated; three of the four females receiving vitamin D cast and reared young whereas none of the vitamin D deficient animals became pregnant. At the end of 138 days the tibia and femor joints of both groups were sectioned and examined by Dr. Gruhzit, head of our Pathology Department.

In the case of the vitamin D deficient animals Dr. Gruhzit reported "the epiphyseal cartilage was well formed with some calcium deposits at the periphery of epiphyseal plate (chondro-osseous separation line). The spongy bone of the shaft and the head were normally developed. The joint line cartilage had a normal appearance. Bone marrow distribution and amounts appeared normal. The animals receiving 500 units per week of irradiated ergosterol likewise were normal."

Animals from the same source were fed the U.S.P. vitamin D deficient diet #2 (*U. S. Pharmacopoeia XIII*) and developed rickets in 22 days as judged by the line test, so we were satisfied the response of the rats receiving the purified vitamin D-free diet was not due to a large storage of the vitamins in the animal's body.

It would appear therefore that vitamin D is not essential to prevent rickets or promote growth in rats receiving a purified diet containing a complete salt mixture. No requirement for this vitamin, therefore, can be given.

4. *Vitamin E*

The vitamin E requirement of the growing rat has been obscured by the large number of studies which have been made using sexually mature rats with the viewpoint of determining the amount necessary for normal reproduction; however, some investigations have been done using the growing rat. Evans *et al.* (1939), Table II, fed 0.75 mg. α-tocopherol daily to 21-day-old male rats and at 277 days found normal seminiferous epithelium in all tubules; at 4 months of age they were mated with normal females and 95% positive matings occurred. Smaller amounts of vitamin E were not fed so a value of 7.5 mg./100 g. (10 g. × 0.75 mg.) of ration may be more than is needed. Mason (1940) has reported that 0.75 mg./100 g. of ration would protect the male and female rat from sterility. In a later paper from Evans laboratory (Nelson *et al.*, 1940) it was found that growth was stimulated in plateaued vitamin E low female rats when

TABLE II

α-Tocopherol Requirements of the Growing Rat

3 Mg./100 g. ration Mg. required/100 g. ration	Investigator
7.5	Evans *et al.* (1939)
0.75	Mason (1940)
2.5–3.5	Nelson *et al.* (1940)
1.0	Hickman (1943)
20–30	Rosenberg (1942)

their diet was supplemented by the daily administration of α-tocopherol at the 0.25 and 0.50 mg. level, which corresponded to 2.5 and 5.0 mg./100 g. of diet. In studying the vitamin A-vitamin E synergism problem Hickman's (1943) data would indicate that 1 mg. of vitamin E gave maximum growth effect when the vitamin A level was sufficiently high.

Summarizing the findings of these four studies it may be concluded that 3 mg. of α tocopherol/100 g. of ration will satisfy the requirements of the growing rat.

5. Vitamin K

There is no evidence that vitamin K is required by growing rats that are fed natural grain or purified diets. Dam (1937) stated that "rats can be raised for a long time without vitamin K." Following ear punching a second generation of 3-week-old rats bled to death and thereafter Jukes (1940) incorporated 2-methyl-1,4-naphthoquinone diacetate in the ration without a recurrence of prolonged bleeding. However, this cannot be taken as proof that rats require vitamin K. Feeding a purified diet that did not contain vitamin K, Richardson *et al.* (1941) noted that the animals grew at a rate similar to the stock colony rats.

It has been reported (Black *et al.*, 1942) and amply confirmed (Kornberg *et al.*, 1944) that incorporating sulfonamides in purified diets will produce a vitamin K deficiency but we are concerned only with regular purified diets. Vitamin K, therefore, can be considered nonessential for the rat.

6. Thiamine

The amount of thiamine required by the rat is a function of several factors—intensity of metabolism, temperature, and composition of the diet.

In one of the early articles on this subject, Arnold and Elvehjem (1938) stated that 80–100 γ of thiamine/100 g. of ration was necessary for growth at a normal rate. It was also found (Light and Cracas, 1938) that the thiamine requirement varied with different strains of rats. One of the three strains tested showed a growth response that was only equivalent to half the response of the other two strains. Using a paired feeding technique in a study of the effect of various B complex deficiencies on appetite and growth, Voris *et al.* (1942) concluded that 100 γ of thiamine/100 g. of ration was insufficient for rat maintenance.

Mature rats receiving a diet containing 64% sucrose and 20% casein required 33 γ/day (330 γ/100 g.) but if the diet contained 64% casein and 19.6% sucrose only 20 γ/day of thiamine was necessary (Wainio, 1942).

Other workers have listed the requirements per 100 g. of ration at

150 γ (McIntire *et al.*, 1943), 100 γ (Elvehjem, 1944; Kline *et al.*, 1945) and 120 to 150 γ (Slanetz, 1943).

TABLE III

Thiamine Requirement of the Growing Rat

125 γ/100 g. ration	
γ Required/100 g. ration	Investigator
80–100	Arnold and Elvehjem (1938)
100	Sure (1940a)
> 100	Voris *et al.* (1942)
150	McIntire *et al.* (1943)
100	Elvehjem (1944)
100	Kline *et al.* (1945)
120–200	Mills *et al.* (1946)

Mills *et al.* (1946) found that 80 γ of thiamine/100 g. of food was mildly inadequate in the cold and markedly so in the heat for weanling rats. Rats 12 months old required 120 γ in the cold and 200 γ in the heat.

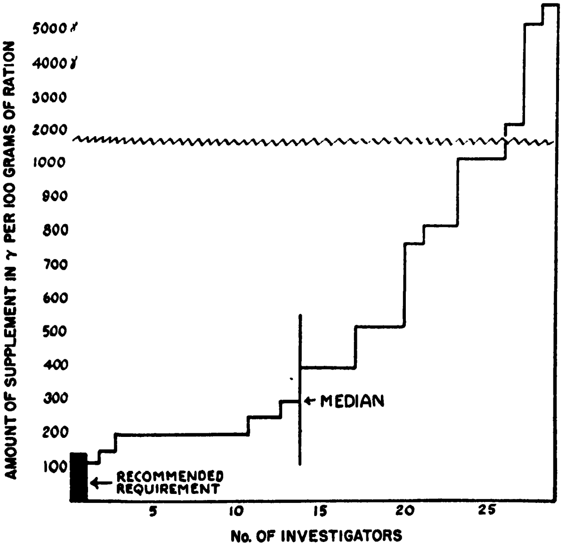

FIG. 1. Amount of thiamine supplement per 100/g. of ration used by 28 investigators.

These requirements, summarized in Table III, range from 80 to 200 γ. From these figures it may be concluded that 125 γ of thiamine/100 g. of ration will supply the minimum requirements for the growing rat.

In Fig. 1 are listed the amounts of thiamine used by 28 investigators selected at random from the literature. It can be observed that the median amount is 400 γ/100 g., and with the exception of two groups all used more than the minimum requirements.

7. *Riboflavin*

The requirements for riboflavin as reported by five investigators are given in Table IV.

TABLE IV

Riboflavin Requirement of the Growing Rat

250 γ/100 g. ration	
γ Required/100 g. ration	Investigator
400	Edgar *et al.* (1937)
130–170	Day and Darby (1938)
120–150	Slanetz (1943)
300	Sure (1940a)
300	Wagner *et al.* (1940)

Edgar *et al.* (1937) were able to obtain maximum growth in depleted rats when 40 γ daily of riboflavin was administered. Using our arbitrary method of assuming a rat eats 10 g. of ration per day this would be 400 γ/100 g. of diet.

Rats receiving approximately 90 to 120 γ/week in a single dose (130–170 γ/100 g. ration) grew to adult size and remained in good health

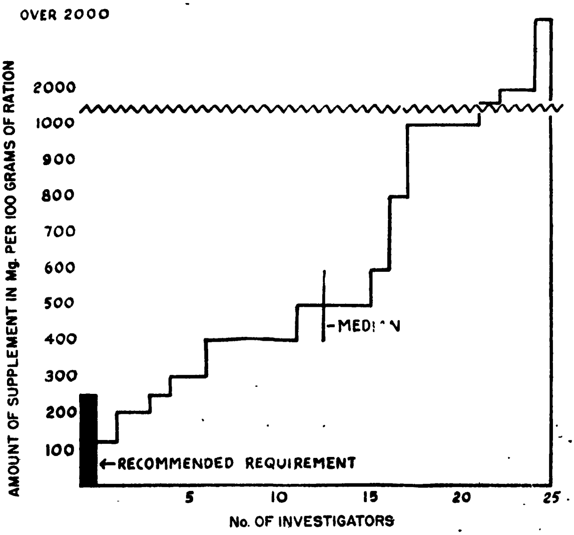

FIG. 2. Amount of riboflavin supplement/100 g. of ration used by 25 investigators.

according to Day and Darby (1938). When similar groups received 30 to 60 γ growth was not sustained to maturity.

Sure (1940a) gives 10 γ (100 γ/100 g. ration) as the riboflavin requirement of the young rat and 30 γ (300 γ/100 g. ration) for the lactating animal. The larger figure has been included in the table since there is no assurance the young rat will attain normal adult weight at the lower level.

Wagner *et al.* (1940), using a purified diet found that animals receiving 30 γ per day (300 γ/100 g. ration) would grow more than 4 g. per day and this is within the normal growth rate of the rat.

The average requirement of these investigators is 250 γ/100 g. of ration. In Fig. 2 are listed the amounts of riboflavin used by 25 investigators selected at random from the literature. Most workers use more than the requirement listed above, the median value being 500 γ/100 g. of ration or approximately twice the minimum value.

8. Pyridoxine

The requirements for pyridoxine as reported by 10 groups of workers are listed in Table V. The majority of them advocate giving approximately 10 γ per day or 100 γ/100 g. of ration.

TABLE V

Pyridoxine Requirement of the Growing Rat

100 γ/100 g. of ration	
γ Required/100 g. ration	Investigator
> 100	Dimick and Schreffler (1939)
100	Conger and Elvehjem (1941)
100	Rosenberg (1942)
100	El-Sadr *et al.* (1939)
100	Kuhn and Wendt (1938)
100	Lepkovsky (1938)
100–250	Sure (1940a)
120–200	Slanetz (1943)
80	Clarke and Lechycka (1943)
> 50	Carpenter *et al.* (1943)

One of the early workers (Lepovsky, 1938) obtained growth with a daily dose of 10 γ per day and dermatitis involving the paws, ears, and areas around the mouth were promptly cured.

Other investigators (Kuhn and Wendt, 1938; El-Sadr *et al.*, 1939) likewise have reported that 10 γ of pyridoxine will satisfy the rat requirement for growth.

Dimick and Schreffler in 1939 reported that 10 γ daily of pyridoxine nearly satisfies the rat requirement. Beef liver extract was used in their studies as a source of unknown vitamin B factors and this may complicate their findings.

Ten to 25 γ daily is recommended by Sure (1940a) for continuous growth.

When riboflavin-depleted rats were fed a purified diet, Conger and Elvehjem (1941) found 10 γ per day would produce a growth of 28 g. per week and Rosenberg (1942), has likewise reported that 10 γ of pyridoxine daily was adequate.

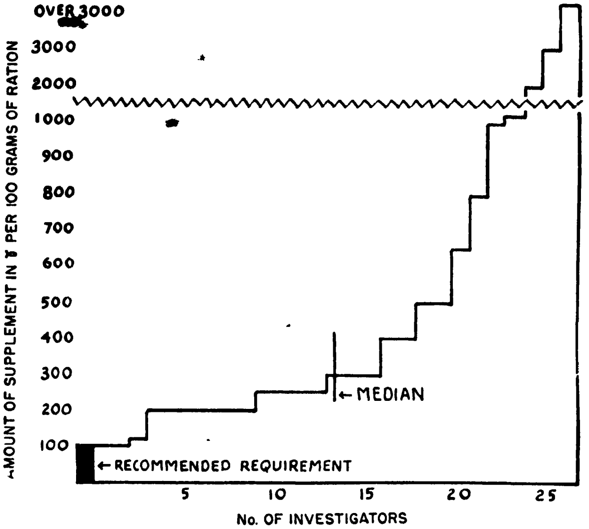

FIG. 3. Amount of pyridoxine supplement/100 g. ration used by 27 investigators.

Other investigators (Carpenter *et al.*, 1943) have found 5 γ per day produced a growth of 24 g. per week. This was below the normal growth of the animal and would indicate a higher dose is required.

Clarke and Lechycka (1943) reported 8 γ per day (80 γ/100 g. ration) produced normal growth for 4 weeks and Slanetz (1943) said "12 γ daily seemed to be optimal for the young growing rat weighing 50 to 120 g. During lactation 120 to 200 γ were needed."

In Fig. 3 has been listed the actual amount of pyridoxine used in their dietary studies by 27 different groups of investigators. It can be seen that all of them use 100 γ or more/100 g. of diet; the median value of this group is 300 γ.

In summary, it would appear that 100 γ of pyridoxine/100 g. of ration will satisfy the growing rat's requirement.

9. Pantothenic Acid

The pantothenic acid requirement of the rat (Table VI) was very easy to obtain because Dr. Unna (1940) made a study of this actual question and later, in conjunction with coworkers, (Unna and Sampson, 1940; Unna and Richards, 1942) made several additional reports which verified his original work. Other investigators (Emerson and Evans, 1941; Elvehjem *et al.*, 1941; Henderson *et al.*, 1942; Bacon and Jenkins,

TABLE VI

Pantothenic Acid Requirement of the Growing Rat

1 Mg./100 g. ration	
Mg. required/100 g. ration	Investigator
0.8	Unna (1940)
0.8–1.0	Unna and Sampson (1940)
1.0	Emerson and Evans (1941)
1.0	Elvehjem *et al.* (1941)
1.0	Henderson *et al.* (1942)
1.0	Unna and Richards (1942)
0.5–1.0	Bacon and Jenkins (1943)
0.8–1.0	Slanetz (1943)
1.0	Rosenberg (1942)

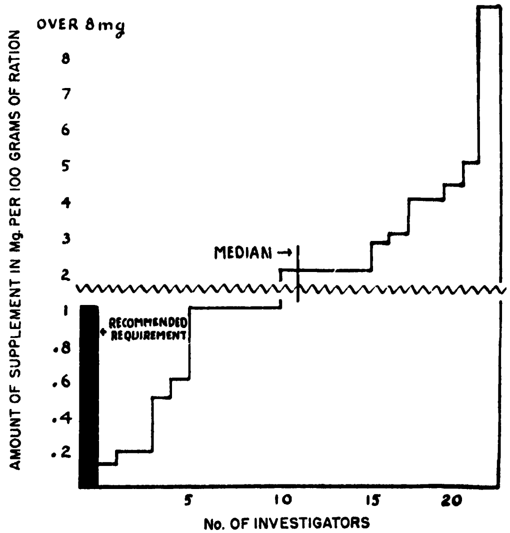

FIG. 4. Amount of pantothenic acid supplement/100 g. of ration used by 22 investigators.

1943; Slanetz, 1943; and Rosenberg, 1942) have confirmed these findings so that the value of 100 γ per day or 1 mg./100 g. of ration is generally accepted as the amount needed by the rat.

One of the interesting phases of the pantothenic acid requirement is that whereas 100 γ/day is necessary for a 3-week-old rat, the need decreases to 25 γ for a 10 week old animal (Unna and Richards, 1942). Very little work has been done with other vitamins at various ages of the rat.

In Fig. 4 it can be seen that 5 of the 22 investigators are using less than 1 mg. of pantothenic acid/100 g. of diet but the median value is 200 γ.

10. Niacin

The value of niacin in the diet of the rat presents a strange situation. Nowhere in the literature were we able to find any evidence that rats fed a purified diet require this material (Table VII), yet from a random survey of 22 investigators (Fig. 5) it was found that all added it to their rat rations with a median value of 5 mg./100 g. Foa *et al.* (1945) have reported that food intake and growth were low when either niacin or niacinamide was used at 0.5% of the diet.

TABLE VII

Niacin Requirement of the Growing Rat

Not required Statement	Investigator
Niacin not a dietary essential	Macrae and Edgar (1937)
Niacin not a dietary essential	Cook *et al* (1937)
Niacin not essential	Birch (1939)
Niacin not necessary for growth	Dann and Kohn (1940)
Rat can synthesize niacin	Shourie and Sweminathan (1940)
Niacin not a vitamin for the rat	Dann (1941)
Niacin synthesized by the rat	Sarett and Perlzweig (1943)
Dietary source of niacin dispensable	Ershoff (1946)

It has been well established that niacinamide functions as a coenzyme that plays an important part in metabolism but since the rat is able to supply its needs without an outside source there does not appear to be any more logic in adding it to the diet than to add any of the many other substances which are produced metabolically.

11. Choline

It was not possible to obtain a uniform value for the choline requirement under all conditions since it varied with the dietary methionine, cystine, betaine, cholesterol as well as the sex, strain, and age of the rat (Griffith, 1940, 1941). In Table VIII it can be noted that the amount reported necessary, per 100 g. of ration, varies from 40 to 150 mg.

Griffith (1941) states that if 30% or more of casein is included in the ration sufficient methionine will be present so that choline can be omitted. Rats maintained on a 24% casein diet apparently have no need for choline, according to Emerson and Evans (1941).

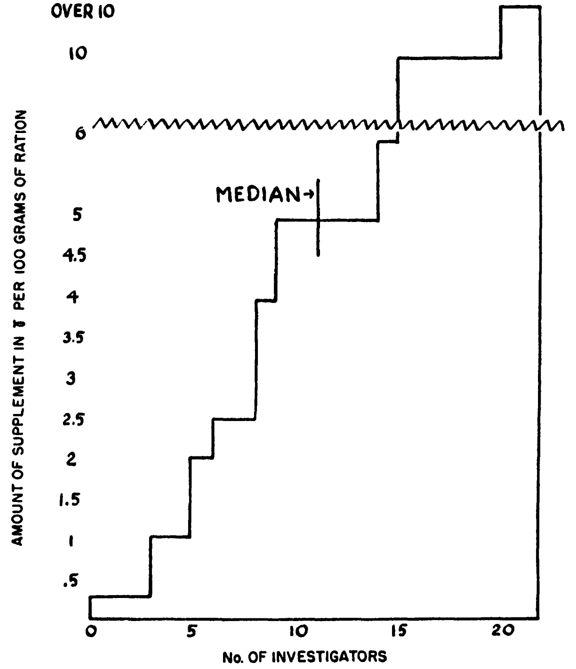

FIG. 5. Amount of niacin supplement/100 g. of ration used by 22 investigators.

There is a decrease in the amount of choline required by rats over 30 days of age. The majority of workers gave 10 to 20 mg. of choline per day to actively growing rats but Slanetz (1943) reported that older rats receiving his purified diet required only 1 to 3 mg. per day.

It was found by Griffith (1940) that deficiency symptoms appeared more slowly and were less severe in young female rats than in male rats of the same age and weight. Hemorrhagic degeneration of the kidneys was prevented in rats receiving a diet containing 18 to 24% casein only when 1 to 2 mg. of choline chloride was included and deposition of liver lipides was prevented by 4 to 6 mg. This requirement for choline in diets containing 24% casein does not agree with the findings of Emerson and Evans reported above but it may be due to strain differences. In this connection Copeland (1944) has developed two strains of rats and found that after the third generation one strain required 4 to 6 mg. of choline per day whereas the other required 10 mg. per day.

TABLE VIII

Choline Requirement of the Growing Rat

100 Mg./100 g. of ration	
Mg. required/100 g. ration	Investigator
60–150	Sure (1940b)
200	Conger and Elvehjem (1941)
200	Patterson and McHenry (1942)
100	Engel (1942)
40–60 (one strain)	Copeland (1944)
100 (second strain)	Copeland (1944)
100–200	Rosenberg (1942)
40–60	Griffith (1940)

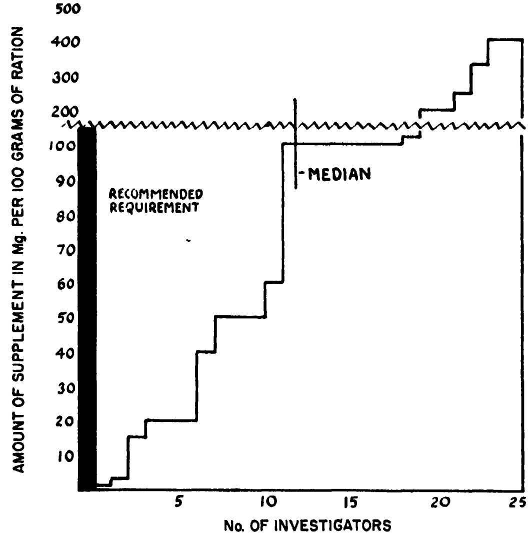

FIG. 6. Amount of choline supplement/100 g. of ration used by 25 investigators.

Figure 6 shows the amount of choline used by 25 investigators and the median value is 100 mg./100 g. of ration. In the case of the other vitamins most people were found to use more than the minimum requirement, but this is not true for choline unless the requirement has been placed too high.

In summary, 100 mg. of choline/100 g. of ration should satisfy the rat requirement when receiving a diet containing 18% casein. However, if the protein is decreased to 10% it will be necessary to increase the choline supplement. With this lower casein intake Patterson and McHenry (1942) found that injecting 20 mg. per day protected the animals.

12. Inositol

Sufficient studies have not been made with inositol to indicate whether it is a required ingredient in the regular purified diet. Pavcek and Baum (1941) reported that the "spectacled eye" syndrome, which had been mentioned previously in the literature, could be cured by administering inositol. These workers gave doses up to 20 mg. daily to effect a cure. Using a natural grain mixture that produced an alopecia in rats, Cunha *et al.* (1943) were able to cure the condition and cause an increase in growth by adding 0.3% inositol to the ration. Other workers have likewise found that loss of hair could be prevented by inositol; biotin, cystine, or methionine also cured this condition (Spitzer and Phillips, 1946).

Ershoff (1944) found that inositol and PABA, in combination or separately at levels of 0.05 to 1.25%, had no effect on growth of rats receiving a purified diet containing six crystalline vitamin B factors—thiamine, riboflavin, pyridoxine, calcium pantothenate, niacin, and choline.

The excellent work that has been done on the prevention and cure of fatty livers with inositol made use of high fat diets which would not normally be used in growth studies (Gavin and McHenry, 1941; Gavin *et al.*, 1943; Engel, 1942; Forbes, 1943; MacFarland and McHenry, 1945). These studies demonstrate the value of inositol under certain dietary conditions but do not help in defining the requirement of inositol for growth of the rat.

In summary, it is not possible to give the inositol requirement of the rat. Apparently the vitamin does no harm in the diet but neither has it any function under ordinary conditions.

13. Para-Aminobenzoic Acid

The state of our knowledge about the requirements for *p*-aminobenzoic acid (PABA) are about the same as for inositol—a few believe it to be an essential while others have not been able to demonstrate its value.

In 1941 Ansbacher reported he was able to correct gray hair in rats by administering 3 mg. of PABA. Emerson (1941), however, could not confirm Ansbacher's findings from the standpoint of preventing or correcting gray hair. She also reported that PABA did not have any influence on the growth response. Work in our laboratory confirmed her results and similar reports were made to the writer by other investigators.

Although we are not concerned with dietary factors necessary for reproduction and lactation in this report, it is of interest that Sure (1941) found PABA to be essential to rats for these functions; he gave 15 mg. daily.

Until more work has been done it would appear that PABA cannot be considered a dietary essential for growth in the rat.

14. Miscellaneous Dietary Essentials

Throughout this review it has been necessary to omit references to those articles which showed that certain materials were essential in the diet if some modification were made, such as unusual quantities of fat or the inclusion of drugs such as the sulfonamides or thyroid.

Using a "standard" diet of purified casein, cerelose, cottonseed oil, minerals, and vitamins it has not been possible to show that folic acid is needed for the normal growth of the rat. However, if sulfa drugs are administered (Endicott *et al.*, 1945) or the animals are fed a low protein diet (Kornberg, 1945) folic acid becomes an essential. No requirements for this vitamin can be given under orindary conditions.

Cary *et al.* (1946) have described a factor present in anti-pernicious anemia liver extract which is necessary for rat growth but to demonstrate the need for this substance requires special alcohol extracted casein in the diet and the mother and young rats fed the ration from birth. It has not been possible consistently to show that this factor is needed under the regular conditions described for the other vitamins.

A new dietary factor called zoopherin has recently been described by the Zuckers (1948). They believe it may be the same as the cow manure factors of Rubin and Bird (1946) and the factor reported by Cary *et al.* (1946). Zoopherin is not concerned with the growth but rather with a decrease in mortality of the young. No requirements for this factor can be given.

Biotin is not a dietary essential when casein is in the diet so it will not be considered in this report.

Recently Novak and Hauge (1948) reported on a growth factor in distillers' dried solubles demonstrable only when a diet was used containing casein that had been extracted with ethanol, dissolved and reprecipitated with acid several times, and finally dehydrated with methanol and acetate. They call the factor vitamin B_{13}. The amount of this substance required by the rat has not been established.

The rat is not dependent upon a dietary source of ascorbic acid. It has not been proven whether this factor is produced by the intestinal flora or manufactured in the tissues. The first possibility could be answered by the use of germ-free animals (Reyniers, 1946).

15. *Discussion*

The proof of the adequacy of the vitamin requirements listed in the review can only be established by long time growth experiments. A preliminary trial using the amounts recommended did not result in growth of 4 to 5 g. per day and even when the vitamin B supplements were doubled the growth rate was not increased. This would indicate that either the fat soluble vitamins, and vitamin A in particular, were given at too small an amount or else one of the B vitamins which we have listed as nonessential is really required.

Once the minimum vitamin requirements are established the investigator will still be faced with the problem of whether to incorporate the minimum amounts or to use an excess. This will depend, of course, on the objective of the study. It was of interest to observe in the random survey of vitamin supplements in current use, that the median amount was often 2 to 3 times the amount found to be required in this review. This increased amount should allow a margin of safety for any deterioration in the ration.

Considerable evidence is now being presented in the literature on factors necessary for pregnancy and lactation in the rat. We have not taken this into consideration in this review. The vitamin requirements of the rat cannot be completely written until these new materials are available in pure form. Then it should be possible to raise rats through several generations with minimum amounts of crystalline vitamins.

16. *Summary*

The vitamin requirements of the rat per 100 g. of ration as established by a literature survey are as follows:

Vitamin A	40 units
Vitamin D	Not required
Vitamin E	3 mg.
Vitamin K	Not required
Thiamine	125 γ
Riboflavin	250 γ
Pyridoxine	100 γ
Pantothenic Acid	1 mg.
Niacin	Not required
Choline	100 mg.
Inositol	Not required
Para-aminobenzoic acid	Not required

II. PARENTERAL VITAMIN REQUIREMENTS

1. *Introduction*

It has been customary for a number of years to inject vitamins whenever there was a question of the utilization by the oral route and although it has been assumed that the response to oral and parenteral administration would be approximately the same—for the water soluble ones at least—very little data were available on this point. While we were in the process of this study an excellent article on the subject was published (Ingelfinger, 1945). Much of this work was with humans, whereas we were dealing solely with the rat.

Generally our procedure with vitamins A, D, thiamine, riboflavin, pyridoxine, and pantothenic acid has been the same, i.e., animals have been maintained on a semipurified deficient diet until they were depleted. They were then given comparable amounts of the missing vitamin by mouth or by injection and the response noted.

2. *Vitamin A*

The details of handling the animals for the vitamin A study were essentially as described in the *U.S. Pharmacopoeia XIII.* The U.S.P. reference cod liver oil, diluted with corn oil, was used as the source of vitamin A. A recheck experiment was performed later using peanut oil as the diluent but since the results were approximately the same only the first experiment will be reported.

TABLE IX

The Vitamin A Growth Response to Parenteral and Oral Treatment with the Reference Cod Liver Oil #2 Dissolved in Corn Oil

No. rats Start	No. rats Finish	Units treated daily	Grams gain or loss in 28 days
		Intramuscular Injection	
20	1	2.04	−21.0
19	4	4.08	− 6.5
		Subcutaneous Injection	
20	2	2.04	− 8.5
20	4	4.08	3.0
		Oral	
20	19	2.04	29.6
20	17	4.08	60.4

Following the vitamin A depletion period 20 animals were started on each level at either 2.04 or 4.08 units daily and the vitamin A was diluted so that the animals received 0.1 cc./day for 28 days. The supplement was given either by mouth, intramuscularly or subcutanously.

The results in Table IX show that the vitamin A deficient animals did not increase in weight following the intramuscular or subcutaneous injection; the majority of them died during the 28 day period and those that lived lost weight except a few animals receiving 4.08 units per day subcutaneously. Autopsies on the animals indicated the oil was not absorbed. In contrast to these results, the animals receiving the vitamin A by mouth made substantial gains. From these results, we conclude that vitamin A dissolved in oil and injected either subcutaneously or intramuscularly was utilized only to a very small extent, if at all. It could be possible that the vitamin A would be absorbed over a longer period of time but during the 28-day test period little if any benefit resulted from the injection. No requirement therefore can be given for vitamin A parenterally.

There is not a general agreement in the literature on the value of parenteral administration of vitamin A dissolved in oil; some investigators have obtained comparable results by the parenteral and oral routes (Lease *et al.*, 1942; Hickman *et al.*, 1944) while others report irregular or poor utilization by the parenteral route (Cienfuegos, 1946; Gounelle *et al.*, 1946; Barlow and Kocher, 1942).

3. Vitamin D

Some clinical investigators have reported that rickets could be prevented or cured by the injection of massive doses of vitamin D (Leake, 1938; Vollmer, 1939; Zelson, 1940; and Vollmer, 1940). In studies on patients with tuberculosis, however, Gounelle *et al.* (1946) found that oral administration of vitamin D_2 caused a rise in serum calcium but with intramuscular injection this did not happen and, if the injection was made at a time when the calcium value was still elevated but beginning to fall after an oral dose, the fall was not interrupted by the injection.

Studies with rats have shown that oral administration is superior to parenteral in oily solutions. The inferiority of injected vitamin D_2 in oil using a prophylatic procedure was explained by the delayed vitamin D absorption from the oily depot in the tissues (Vollmer and Oser, 1947).

To obtain more information on the response of rachitic rats to the parenteral and oral administration of vitamin D_2 dissolved in oil we fed young animals the Steenbock-Black rachitogenic diet for 21 days, following the procedure described in the *U.S. Pharmacopoeia XIII*. The presence of rickets was established by line testing a representative number of the animals. Comparable groups were then assembled and the U.S.P. reference cod liver oil, diluted with corn oil, was administered either by intramuscular injection or by mouth daily for 7 days. On the eighth day the animals were killed, the leg bone removed and the degree of recalcification observed.

The results in Table X indicate that vitamin D is utilized by intramuscular injection but only approximately half as efficiently as by oral administration during the period of the test. This confirms the clinical work described above from the viewpoint of utilization; it indicates also that the oral route would produce a prompter healing of the rachitic bone. The oil solution could permit the vitamin D to act over a longer period of time.

TABLE X

The Vitamin D Response to Parenteral and Oral Treatment with Reference Cod Liver Oil Dissolved in Corn Oil

No. rats	Total units Administered	Degree of recalcification
	Intramuscular	
14	4.6	1.0
13	5.75	1.2
	Oral	
14	4.6	1.9
14	5.75	2.0

The data obtained from the vitamin D study are in marked contrast to the vitamin A injection experiment. Even though this latter test was conducted for 28 days the biological response was negligible. It is known that the sex hormones are absorbed from oily solutions and the fact that the vitamin D molecule is similar to them may explain why vitamin D is utilized to a greater extent than vitamin A. Unpublished data in our laboratory indicate that aqueous solutions of vitamins A and D given parenterally are utilized as well as or better than oil solutions given by mouth (Brown, 1948).

In summary the amount of vitamin D required when oily solutions were used was 100% greater when it was administered parenterally than when given orally.

4. Thiamine

Of all the B vitamins which are injected in humans thiamine is the most widely used. A summary of these studies has been made by Ingelfinger (1945).

In our comparison of the growth response of rats receiving the vitamins by the oral and parenteral routes the same basal diet was used for all the B vitamins, omitting the particular one under study. The complete purified diet consisted of purified casein, 18%; cerelose, 68%; cottonseed oil, 5%; corn oil, 3%; cod liver oil, 2%; salt mixture (Jones and Foster, 1942), 4%; and the following vitamin B factors per 100 g. of ration: thiamine, 0.8 mg.; riboflavin, 0.8 mg.; pyridoxine, 0.8 mg.; sodium pantothenate, 10 mg.; niacin, 10 mg.; inositol, 10 mg.; p-aminobenzoic acid, 10 mg.; and choline chloride, 60 mg. Using the above diet, omitting thia-

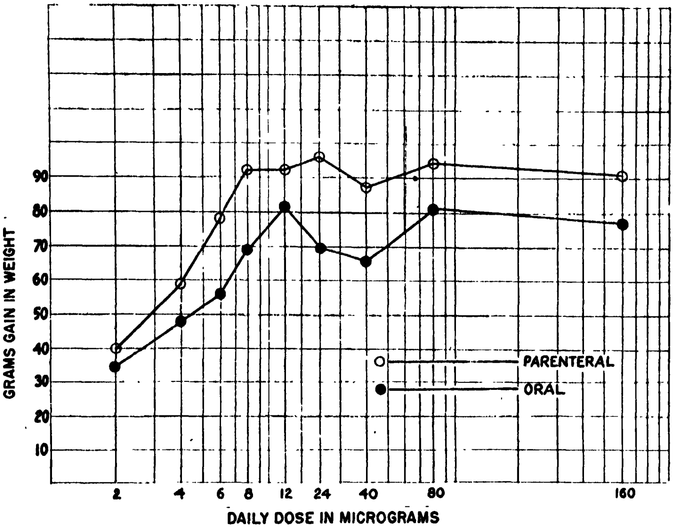

FIG. 7. Gain in weight in 6 weeks of rats receiving thiamine parenterally and orall.

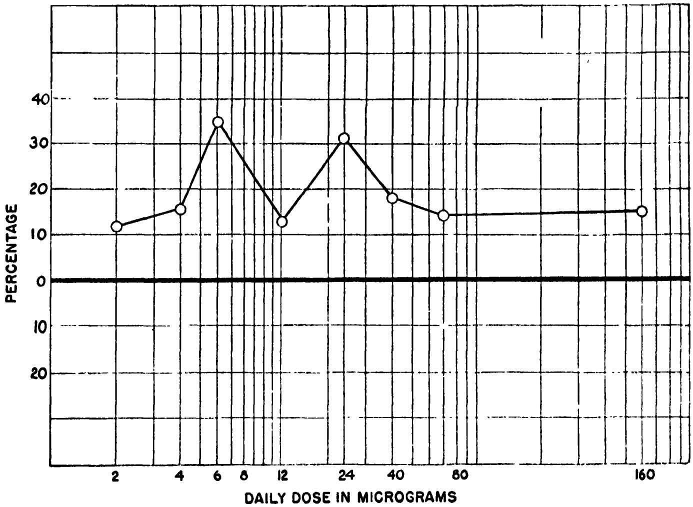

FIG. 8. Percentage increase in 6 weeks of body weight of rats receiving thiamine parenterally over rats receiving thiamine orally.

mine, weanling rats were depleted in an average of 28 days as evidenced by stationary or declining weight. The animals were then placed in comparable groups of approximately 12 each, care being taken to distribute the sexes and weights equally. The respective groups were treated either orally or intramuscularly with 2, 4, 6, 8, 12, 24, 40, 80, and 160 γ over a 6-week period.

The final gains in weight together with the percentage increase of the intramuscularly injected over the orally treated animals are shown in Figs. 7 and 8. Dr. R. C. Miller of the Pennsylvania State College very kindly analyzed the data statistically for thiamine, riboflavin, pyridoxine, and pantothenic acid. In the case of thiamine he found the differences between the two routes of administration to be highly significant. However, the data would not permit a calculation to show how much better the parenteral treatment was over the oral. It is of interest to observe that a maximum growth was obtained at the 8 γ dose for the parenteral route and at 12 γ for the oral. No explanation can be given for the drop in weight at the oral doses of 24 and 40 γ.

The differences between the oral and parenteral results may be explained by the instability of thiamine in the more alkaline gastrointestinal tract when gastric secretion is at a minimum (Melnick *et al.*, 1941).

These results indicate that the thiamine requirement when given by intramuscular injection is less than when given orally.

5. *Riboflavin*

The results following oral or subcutaneous injections of riboflavin are similar to those found for thiamine. The animal procedure was comparable to the preceding report; rats weighing 45 to 55 g. were fed a riboflavin deficient diet until there was a decline in weight; this occurred in an average of 25 days. Riboflavin was then administered at doses of 2, 4, 6, 8, 12, 24, 40, and 80 γ for a period of 6 weeks and the gains in weight compiled. This experiment was completed twice and since the data were similar they were combined. Each point on the graph represents approximately 30 rats.

The results in Figs. 9 and 10 show that the animals receiving riboflavin by the parenteral route, with but one exception, gained more than comparable groups of animals receiving the vitamin orally. Dr. Miller has analyzed the results statistically and found a significant difference. As in the case of thiamine this difference may be due to a slight destruction of riboflavin in the intestinal tract.

It would appear from these data that the riboflavin requirement of

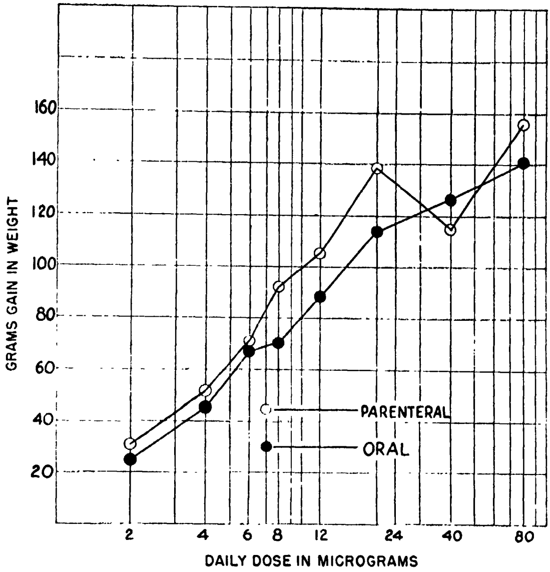

FIG. 9. Gain in weight in 6 weeks of rats receiving riboflavin parenterally and orally.

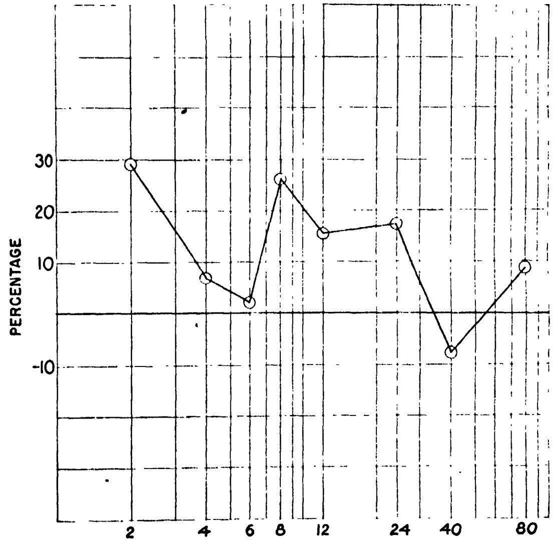

FIG. 10. Percentage increase in 6 weeks of body weight of rats treated with riboflavin parenterally over rats receiving riboflavin orally.

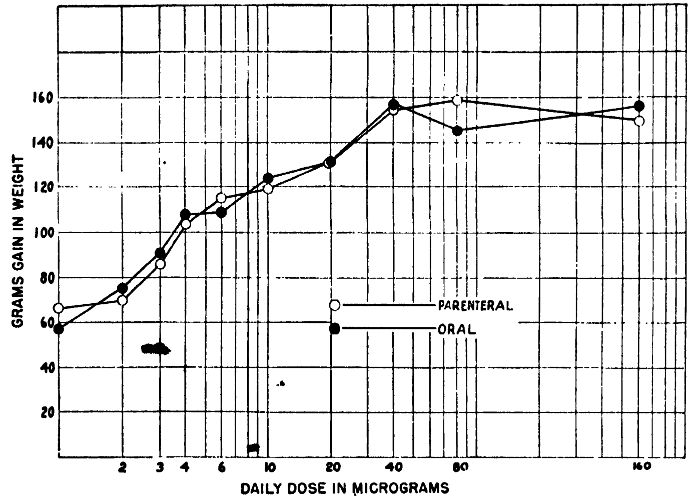

FIG. 11. Gain in weight in 6 weeks of rats receiving pyridoxine parenterally and orally.

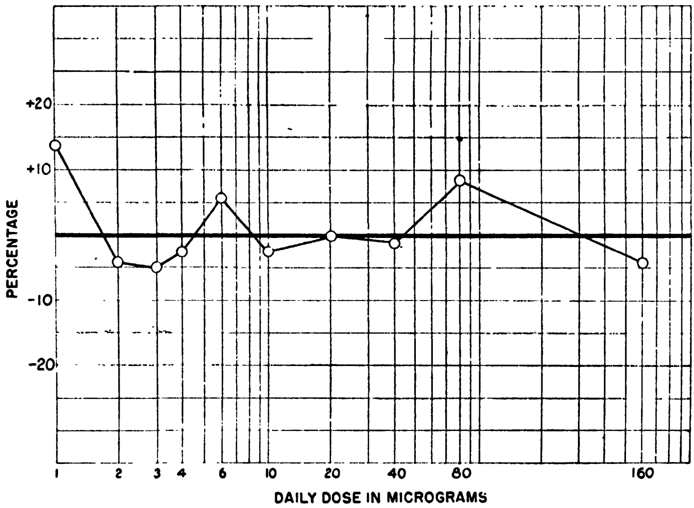

FIG. 12. Percentage increase in 6 weeks of body weight of rats treated with pyridoxine parenterally over those treated orally.

the rat is less when the material is given subcutaneously than when given orally.

6. *Pyridoxine*

In comparing the growth response to pyridoxine given orally and subcutaneously, rats weighing 40 to 55 g. were fed the purified diet listed above under thiamine, for 56 days. They were then arranged in comparable groups of approximately 15 rats each and doses of 1, 2, 3, 4, 6, 10, 20, 40, 80, and 160 γ were administered over a 6-week period and the weight gains recorded.

The results given in Figs. 11 and 12 show that there is no difference between the oral and the parenteral utilization of this vitamin. There is a difference between the two groups of approximately 10% but this could be considered normal for the test. It is also of interest to observe from the graph showing the increase in weight that the gains of both groups reach a maximum at the level of 40 γ/day. Assuming that the rat eats 10 g./day, this would amount to 400 γ/100 g. of ration which is considerably above our estimated requirement from the literature of 100 γ/100 g. of ration. However, in our work, we were using depleted rats and the requirement may be higher than when the vitamin is given prophylactically.

To summarize, the requirement of the rat for pyridoxine is the same orally and parenterally.

7. *Pantothenic Acid*

The procedure for studying the oral and subcutaneous requirements for pantothenic acid was similar to that used for the other B vitamins. The rats were started on treatment after receiving the deficient diet for 36 days.

The results in Figs. 13 and 14 show that no difference in the growth response from the oral and parenteral methods of administration was obtained. The animals reached a plateau in their weight gain at between 80 and 120 γ/day which by calculation would be 0.8 to 1.2 mg./100 g. of ration. This agrees with the estimated requirement of 1 mg./100 g. of ration.

The pantothenic acid requirement is the same whether given orally or subcutaneously.

8. *Summary*

We can conclude from these studies that vitamin A dissolved in oil is utilized only to a very slight extent, if at all, when injected subcutaneously or intramuscularly; vitamin D is utilized 50% as efficiently by intramuscular injection as by oral administration.

Thiamine and riboflavin are more effectively utilized when given

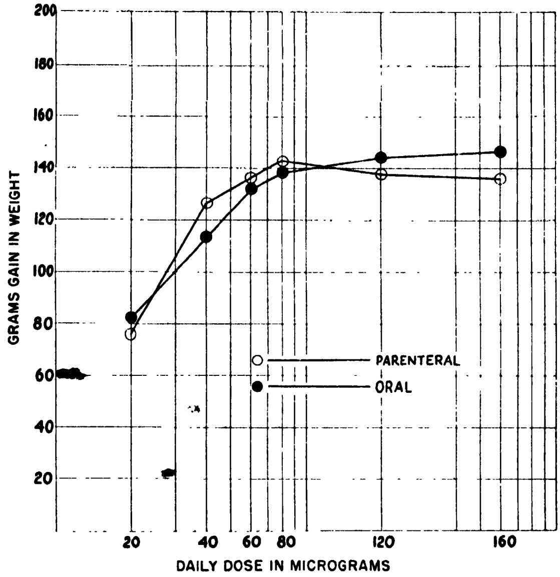

FIG. 13. Gain in weight in 6 weeks of rats receiving pantothenic acid parenterally and orally.

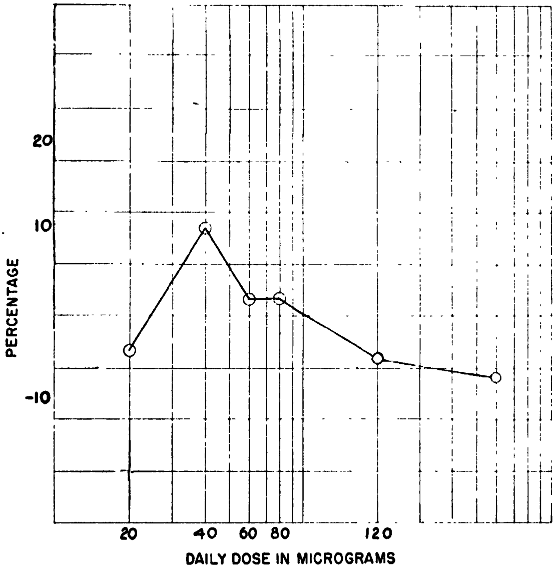

FIG. 14. Percentage increase in 6 weeks of body weight of rats treated with pantothenic acid parenterally over rats receiving pantothenic acid orally.

parenterally whereas there is no difference in the growth response to pyridoxine and pantothenic acid whether given parenterally or orally.

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Vitamin Requirements of the Guinea Pig*

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I. INTRODUCTION

The guinea pig has been employed as a laboratory animal since the early part of the nineteenth century (Mason, 1940) and was one of the first species in which a vitamin deficiency was induced experimentally when Holst and Frölich (1907) recognized scurvy in this herbivore. Yet today little is known of the vitamin requirements of the guinea pig. An examination of the literature reveals only meager and often inconclusive evidence for the production of deficiencies of the various members of the vitamin B complex. Acute vitamin A and E deficiencies have been repeatedly demonstrated, but the quantitative needs for these vitamins have not been defined. Only the requirement for ascorbic acid has been determined accurately.

The realization that so little is known about guinea pig nutrition often comes as a surprise to workers in the field who have assumed, quite naturally, that a laboratory animal of such long standing and wide acceptance would have been thoroughly studied. Part of this lack of information is due to the emphasis placed on the rat with the consequent neglect of other species. The rat was early selected as the animal of choice for nutrition studies, and in retrospect, we know this selection to have been a wise one, since, in addition to the many manifest qualities of the rat which suit it for nutritional studies, we now realize that its vitamin needs are less complex than those of most other species eligible as laboratory subjects. The fact that under proper conditions the growing rat does not require a dietary source of ascorbic acid, vitamin K, niacin, biotin, or pteroylglutamic acid minimized the effort needed in establishing its vitamin requirements.

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Disinterest alone, however, does not account for the lack of knowledge of guinea pig nutrition. Rather, certain inherent qualities of the guinea pig itself render it a difficult subject for nutritional investigation: 1) The guinea pig is more specialized in its dietary requirements than most other species that have been studied. It is remembered that only the guinea pig and the primates are known to require a dietary source of ascorbic acid throughout their life cycles. Further, it is generally agreed that the crystalline vitamins do not satisfy all of the vitamin requirements of the guinea pig. The problem has been one of devising diets deficient in a given vitamin while still supplying the unknown essentials. 2) The guinea pig is extremely susceptible to bacterial infection. While this characteristic has been exploited to great advantage in the field of bacteriology, it has tended to discourage nutritional investigation. 3) Guinea pigs are notorious coprophagists. Although the regular pseudorumination cycle of the rabbit has not been observed in the guinea pig, it has been suggested that a less systematic cycle of feces-eating prevails (Winterstein, 1911). Feces have come to be recognized as excellent sources of many of the B complex vitamins, and a great part of the variability encountered in the growth of guinea pigs receiving experimental diets may well be due to the degree of coprophagy practiced by individuals. 4) With respect to birth weight and growth rate guinea pigs lack genetic uniformity. Breeding has been directed toward the development of strains showing increased or decreased resistance to certain infectious diseases, fecundity, or perfection of special characteristics of esthetic interest, not toward the establishment of animals responding in a like manner to a given dietary regimen. In short, the guinea pig is poorly adapted to nutritional research.

II. Ascorbic Acid

Being the only small laboratory animal available for ascorbic acid studies, the guinea pig has received careful attention regarding its requirement for this vitamin. The vitamin requirements of laboratory animals have usually been derived from assay curves obtained through employment of the prophylactic method, and therefore it has become customary to express the minimal requirement for a given vitamin as the smallest quantity necessary for the promotion of maximal growth or the prevention of characteristic deficiency symptoms in young, growing animals maintained under restricted experimental conditions for limited periods of time. This does not represent the many requirements that exist. In addition to the requirements of the growing animal there are requirements for the maintenance of the adult, for reproduction, for lactation, and for the aged animal. Furthermore, external factors such as environmental temperature, exercise, the type or amount of ration being fed, mode and frequency of administration of the vitamin, and duration of the test period may be expected to affect the quantitative, and even the qualitative, need for the vitamin. Almost all of our knowledge of the ascorbic acid requirement of the guinea pig has been gained from the use of young animals over relatively short experimental periods, and even here, where conditions were comparatively well standardized, minimal requirements have been expressed in a variety of ways: the requirement for growth, for protection against macroscopic scurvy, for the prevention of microscopic scurvy, for optimal growth of odontoblasts, for wound healing and bone regeneration, for maintenance of the maximum serum phosphatase level, for saturation of the tissues with ascorbic acid. It is seen in Table I that while a daily dose of 2 mg. of ascorbic acid satisfies most of these "requirements," still other minimal levels may be defined depending upon the criterion of deficiency being considered.

Soon after isolating ascorbic acid Waugh and King (1932a, b) showed that 0.5 mg. of the vitamin daily would protect a majority of guinea pigs against the development of macroscopic signs of scurvy. Despite the fact that detection of incipient scurvy depends largely on the judgment and skill of the laboratory worker, rather good agreement with this figure was obtained by other investigators (Svirbely and Szent-Györgyi, 1933; Randoin, 1934; Göthlin, 1934; Djabri, 1940; Dunker *et al.*, 1942; Crampton *et al.*, 1944, 1947a).

As first demonstrated by Zilva and Wells (1919), the earliest manifestations of ascorbic acid deficiency are changes in the microscopic structure of the roots of the incisor teeth. These changes, particularly of the parenchymal cells, appear much earlier in the course of deficiency than the more commonly observed macroscopic signs of scurvy, and larger amounts of ascorbic acid are required for their prevention. Using this sensitive measure of deficiency, Harris and Ray (1933) and Key and Morgan (1933) found 2 mg. of ascorbic acid daily to be necessary for maximum protection. Calculated on a body weight basis, Kuether *et al.*

TABLE I

Ascorbic Acid Requirement of the Guinea Pig

Criterion of deficiency	Daily requirement[1] (mg.)	Author
Growth	0.4	Dawbarn, 1945
	0.6	Dunker *et al.*, 1942
	1->1	Cohen, 1940; Coward and Kassner, 1936; Djabri, 1940; Randoin, 1934; Swachman and Gould, 1942
	2	Crampton *et al.*, 1944
Macroscopic scurvy	0.5	Crampton *et al.*, 1944; Dunker *et al.*, 1942; Djabri, 1940; Göthlin, 1934; Randoin, 1934; Svirbely and Szent-Györgyi, 1933; Waugh and King, 1932b
Microscopic scurvy	1.3	Göthlin, 1934
	2	Harris and Ray, 1933; Key and Morgan, 1933
	2.4	Hou, 1936a
	> 2.5	Cohen, 1940
	1[2]	Kuether *et al.*, 1944
Odontoblast growth	2	Crampton *et al.*, 1944
Wound healing	2	Bourne, 1942a, 1944a
Bone regeneration	2[3]	Bourne, 1942b, 1944b
Serum phosphatase	0.23	Gould and Shwachman, 1943
Reproduction	2-5	Crampton and Bell, 1947b
Prolonged survival	5 or less	Zilva, 1936
Tissue saturation	25	Zilva, 1936; Penny and Zilva, 1946
	50 or more	Giroud *et al.* 1935, 1937, 1938, 1943

[1] Per animal (oral) unless otherwise indicated (animals weighing 250-350 g. were customarily employed).

[2] Per 100 g.

[3] Parenteral.

(1944) obtained the somewhat higher value of about 1 mg. of ascorbic acid per 100 g. of animal. Hou (1936a) found 2.4 mg. of ascorbic acid daily to be necessary for tooth protection, and Cohen (1940) did not obtain complete freedom from microscopic scurvy in animals receiving as much as 2.5 mg. per day. Examining molar rather than incisor tooth sections, Göthlin (1934) observed microscopic alterations at a 1.0 mg. per day level but not at a daily intake of 1.33 mg. Crampton *et al.* (1944) measured the length of odontoblasts of guinea pigs receiving graded doses of ascorbic acid and concluded that maximum length can be achieved with a 2 mg. daily dose of the vitamin.

The minimal requirement of ascorbic acid for growth varies greatly as recorded in the literature. Dawbarn (1945) observed as good growth with 0.4 as with 0.8 mg. of ascorbic acid daily, and Dunker *et al.* (1942) obtained a maximum growth response with 0.6 mg. per day. Others

(Randoin, 1934; Coward and Kassner, 1936; Djabri, 1940; Cohen, 1940; Shwachman and Gould, 1942) found the requirement to approach or to exceed 1 mg. daily. Growth, being a nonspecific sign of ascorbic acid deficiency, would be expected to reflect other inadequacies of the diet than ascorbic acid as optimal levels of this vitamin were being approached in the diet, and the minimal ascorbic acid requirement for growth would then vary with the degree of general adequacy of the diet being employed. Crampton (1947a) studied some of the diets used by the above investigators and found them entirely incapable of promoting normal growth of guinea pigs to maturity or of supporting successful pregnancy. Employing an improved scorbutic diet, Crampton *et al.* (1944) found a daily intake of 2 mg. of ascorbic acid to more nearly satisfy the minimal needs of the guinea pig for growth.

Bourne (1942a, 1944a, 1946) studied the effect of ascorbic acid on the degree of healing of experimental skin wounds in guinea pigs. Optimal healing was secured with 2 mg. of ascorbic acid daily. In similar studies where the rate of bone regeneration was being measured, Bourne (1942b, 1944b, 1946) again found 2 mg. of ascorbic acid daily to be the optimal dose. However, in the latter work ascorbic acid was administered parenterally, which, as will be discussed later, significantly enhances utilization of the vitamin. The strength of guinea pig gastrocnemius muscle bears a relationship to the ascorbic acid intake, a maximum response being obtained with a daily intake of 2.5 mg. (Hines *et al.*, 1944).

The serum phosphatase level is known to be greatly reduced in scurvy, and Gould and Shwachman (1943) have shown 0.225 mg. of ascorbic acid per day to be the critical dose necessary for maintenance of the normal phosphatase content of guinea pig serum.

The minimal ascorbic acid requirement for reproduction or prolonged survival has not been worked out in detail, but Zilva (1936) has kept guinea pigs in a healthy state for a number of years on a daily intake of about 5 mg. of ascorbic acid. Crampton and Bell (1947b) find 2 to 5 mg. of ascorbic acid per day satisfactory for reproduction.

Marsh (1936) and Horgan (1936) described a reduction of serum complement titer associated with ascorbic acid deficiency, and Ecker and Pillemer (1940) state that 10 to 20 mg. of ascorbic acid must be fed daily to assure a normal complement value in guinea pigs. At the time, these observations offered an explanation for the well-known susceptibility of scorbutic animals to infection, but unfortunately, subsequent investigation has failed to substantiate any relationship between ascorbic acid intake and serum complement level (Zilva, 1936; Maccolini, 1939; Agnew *et al.*, 1942; Kodicek and Traub, 1943).

The validity of employing the saturation method as a measure of the adequacy of ascorbic acid nutrition in humans has been challenged in studies using the guinea pig. Zilva (1936) and Penney and Zilva (1946) showed that about 25 mg. of ascorbic acid must be administered daily to insure saturation of the tissues with the vitamin. The degree of previous tissue saturation was found to have little to do with the length of survival of animals fed a scorbutic diet; the time taken for the animals to succumb to scurvy was independent of the pre-experimental intake of ascorbic acid as long as it exceeded 1 mg. per day. During depletion there is a rapid decrease in ascorbic acid concentration of the tissues for the first 8 days, and then the loss becomes progressively smaller. These observations suggest that the fraction of tissue ascorbic acid lost in the initial stages of depletion does not act as a store for protection against scurvy, that saturation is in reality physiological "supersaturation." In view of the minimal ascorbic acid levels found necessary for protection against scurvy, for reproduction, and for prolonged survival, there is little to support the concept that saturation with ascorbic acid is a prerequisite of adequate nutrition. Giroud *et al.* (1935, 1937, 1938, 1943) found a daily intake of 50 mg. or more of ascorbic acid to be necessary for tissue saturation. A slight beneficial effect of previous saturation on length of survival was also noted (Giroud *et al.*, 1943).

The mode of administration is known to influence the ascorbic acid requirement of the guinea pig. Grollman and Firor (1934) found ascorbic acid to be much more effective in protecting against scurvy when given intraperitoneally than when fed. Hou (1935, 1936b) showed the minimal protective dose of ascorbic acid by oral administration to be twice that by subcutaneous injection, and Gould and Shwachman (1943) in comparing the effectiveness of oral and parenteral administration, calculated a loss of 27% of the vitamin when it was given by mouth. A single dose of 25 mg. of ascorbic acid, which is adequate for saturation of the tissues when given parenterally, fails to effect saturation when given orally (Penny and Zilva, 1946). Similar results had been reported earlier by DeCaro (1936).

Since tissue stores of ascorbic acid are rapidly depleted, it would be expected that the rate of administration of the vitamin would have considerable bearing on the requirement, and Zilva (1941) has reported that guinea pigs receiving 2 mg. of ascorbic acid every day appeared normal while those given a single weekly dose of 14 mg. showed inferior growth, and, in some animals, slight signs of scurvy. However, no disadvantage in economy of ascorbic acid utilization for prevention of scurvy results when the vitamin is given on alternate days or on every third day (Hou, 1936b; Zilva, 1941). The rate of administration greatly affects the degree of tissue storage of ascorbic acid (Jacobsen, 1935; Penney and Zilva, 1946). When 25 mg. of ascorbic acid is given orally in a single dose, the ascorbic acid content of the tissues is less than half that obtained when 25 mg. is given in 50 doses at 10 minute intervals (Penney and Zilva, 1946).

III. Vitamin A

Numerous investigators have shown the guinea pig to be extremely sensitive to a deficiency of vitamin A and have studied the pathological aspects of the disease in detail (Tozar, 1921; Boock and Trevan, 1922; Wolbach and Howe, 1928, 1933; Hetler, 1934; Bentley and Morgan, 1945). These are not unlike these observed in other species. Yet in the above studies no attempts were made to determine the carotene or vitamin A requirement. At birth the stores of vitamin A are very low, but this incurs no serious disadvantage under natural conditions since the guinea pig begins eating green foods soon after it is born. But even while consuming diets rich in carotene, the guinea pig shows little tendency to store vitamin A (Simonnet *et al.*, 1932; Clausen and McCoord, 1934; Chevallier and Choron, 1935, 1936, 1937; Bentley and Morgan, 1945). This may be considered somewhat surprising in an animal whose herbiverous habits limit its intake of vitamin A to the provitamin form. Clausen and McCoord (1934) state that only 6% of a massive dose of vitamin A can be recovered from the liver of the guinea pig as compared to a recovery of 71% from the liver of the rat. While the rat may store enough vitamin A in a few days to satisfy its nutritional needs for months (Baumann *et al.*, 1934; Davies and Moore, 1937), it would appear that the guinea pig must rely on rather frequent intake of the vitamin. However, not all guinea pigs share this inability to convert carotene or to store vitamin A. Chevallier and Choron (1935, 1936, 1937) found among apparently normal animals receiving a carotene-rich diet, guinea pigs whose liver stores of vitamin A compared favorably with those of rats, while the reserves of vitamin A in others were negligible. The basal metabolic rate of animals having abundant stores of the vitamin was considerably below that of those not possessing such reserves (Chevallier and Jullien, 1938), an unexpected finding in view of the evidence in other species of a specific role of thyroxine in the conversion of carotene to vitamin A (Johnson and Baumann, 1947). It has been observed, however, that the administration of thyroxine prevents the deposition of carotene or its transformation to vitamin A in guinea pigs (Abelin, 1933). The ability of a guinea pig to transform carotene or to store vitamin A may well be a function of its thyroid activity.

IV. Vitamin B Complex

What little is known of the requirements of the guinea pig for members of the vitamin B complex can be summarized in a few brief statements.

Thiamine deficiency has been produced in guinea pigs (Carlström and Jonsson, 1939; Lilleengen, 1940; Pellegrino and Pinho, 1945) but the minimal requirement has not been determined.

Slanetz (1943) states that the guinea pig requires about 300 γ of riboflavin per 100 g. of ration.

Morgan and Simms (1940) have described what may have been a pantothentic acid deficiency in the guinea pig. Three of four black animals on a deficient diet showed a dullness and metallic appearance of the fur while a fourth turned completely gray. It is not certain that the achromotrichia was due to a lack of pantothenic acid since a crude preparation of the vitamin failed to restore hair coloration or the general condition of the gray animal.

Harris (1939) concluded that niacin is a dietary essential for the guinea pig. The mortality rate of animals was lowered and the growth improved when niacin was added to a modified Goldberger diet. Worden and Slavin (1944) obtained inconclusive results in similar experiments. Following the pattern of the experiments of Krehl *et al.* (1945a, b), who have shown that a niacin deficiency can be produced in rats if the tryptophan intake is sufficiently low, Cannon *et al.* (1946) obtained similar, but less dramatic, results in the guinea pig. Handler (1944) has pointed out a marked difference in the manner in which rats and guinea pigs excrete large doses of niacinamide. High dietary levels of niacinamide are toxic to the rat but not to the guinea pig. This is explained by the fact that rats excrete niacinamide as N^1-methylniacinamide, a process depleting the animal of its labile methyl reserves, while in guinea pigs no increase in the excretion of N^1-methylniacinamide is found when excessive amounts of niacinamide are fed.

Woolley and Sprince (1944, 1945) prevented a folic acid deficiency in a small group of guinea pigs with the daily feeding of 6.5 γ of folic acid (pteroylglutamic acid). In this laboratory we have also recognized the need of the guinea pig for folic acid but find that as much as 100 γ daily are required before a maximal response is obtained (Mannering and Brown, 1946). On the other hand, the Wisconsin workers have never been able to demonstrate a folic acid deficiency in the guinea pig (Mannering *et al.*, 1943; Booth and Elvehjem, 1948). While such divergencies between laboratories are difficult to explain, it should be mentioned that although a majority of guinea pigs receiving a folic acid low diet fail to grow or survive in our experiments, others develop in a manner approaching normal. No other explanation being readily available, coprophagy or refection best serves as a rationalization for this variation.

An attempt by Lease *et al.* (1937) to induce a biotin deficiency in the guinea pig by feeding raw egg white yielded inconclusive results.

Hogan and Hamilton (1942) noted some beneficial effect of inositol in guinea pigs fed purified diets.

No account was found in the literature of a pyridoxine or choline deficiency ever having been observed in the guinea pig.

V. VITAMIN D

The widespread belief that the guinea pig could not develop rickets persisted until Emerique (1937) induced high calcium:low phosphorus rickets in this species and effected cures with unspecified amounts of irradiated ergosterol. Low calcium: high phosphorus rickets has also been observed (Harris, 1940; Howe *et al.*, 1940). However, when fed a vitamin D low ration in which the salt mixture is assumed to have been balanced, guinea pigs have been maintained for 3 months without becoming rachitic (Kodicek and Murray, 1943). It is quite probable that, like the rat, the young guinea pig does not require vitamin D when the proper calcium:phosphorus ratio of the diet is preserved.

VI. VITAMIN E

The symptoms of vitamin E deficiency, characterized by extensive skeletal muscle dystrophy, were first observed in the guinea pig by Goettsch (1930) and by Pappenheimer (1930). They believed the condition resulted from a lack of some factor other than vitamin E (Goettsch and Pappenheimer, 1931), and although the syndrome was studied by others for several years (Rogers *et al.*, 1931; Madsen *et al.*, 1933, 1936; Wood and Hines, 1937), this idea persisted until Olcott (1938) pointed out the similarity of the skeletal muscle lesions found in young rats obtained from vitamin E deficient mothers to the nutritional muscle dystrophy seen in guinea pigs and rabbits. Finally, Shimotori *et al.* (19,9) showed that pure α-tocopherol would completely protect guinea pigs. Protection for as long as 200 days was afforded with 1.5 mg. of the pure compound per day. Single doses of 25 mg. bring about rapid regression of the lesions (Pappenheimer, 1943). Weekly doses of 5 or 10 mg. prevent muscular dystrophy in pregnant guinea pigs but are inadequate for protection against resorption of the fetuses (Pappenheimer and Goettsch, 1941).

VII. VITAMIN K

Dam *et al.* (1937) were unable to detect any signs of deficiency in young guinea pigs fed a vitamin K deficient diet during an observation period of 6 weeks. On the other hand, Hamilton (1943) has shown that when vitamin K is withheld from the diet of pregnant animals, the young are stillborn or die shortly after birth with extensive subcutaneous, intramuscular, and brain hemorrhages. Fifty milligrams of 2-methyl-1,4-naphthoquinone per kilogram of ration protected the guinea pigs. Lesser amounts were not fed.

VIII. Vitamin P

Szent-Györgyi and his collaborators (Armentano *et al.*, 1936) reported the existence of a substance other than ascorbic acid which could control the number of hemorrhages occurring in certain clinical conditions. This substance, originally obtained from Hungarian red-peppers and lemon juice, and later shown to be present in a variety of plants, was called "Citrin" or "vitamin P." The latter term is now used collectively to include a whole series of physiologically active flavones and their glycosides. Citrin was shown to be a mixture of hesperidin and eriodictyol glucoside (Bruckner and Szent-Györgyi, 1936). Scorbutic guinea pigs responded to vitamin P with a rise in capillary resistance and scurvy was thought to be a codeficiency of ascorbic acid and vitamin P (Bentsáth *et al.*, 1936, 1937). The status of vitamin P as a vitamin was improved when it was shown that the imposition of an ascorbic acid deficiency was not an obligatory requisite for the demonstration of vitamin P effects (Zacho, 1939; Rusznyák and Benko, 1941; Bacharach *et al.*, 1942). Nevertheless, there has been considerable confusion in the devising of a biological assay for vitamin P. Many investigators, particularly in this country, have been unable to show any vitamin P effect. Others, including Szent-Györgyi himself (1938), have failed at times to repeat their work. Since guinea pigs appear to thrive without vitamin P, there is some question as to whether these compounds should be classified as vitamins or as pharmaceuticals. Recently, however, Bhagvat (1946) noted a slight stimulatory action of hesperidin on the growth of guinea pigs.

Of the compounds possessing vitamin P activity, hesperidin and eriodictin have received the most attention, but it has been recognized almost from the beginning that their activities are highly inferior to those of the crude materials from which they are obtained. The flavanone, epicatechine, is known to be about 500 to 1000 times more active than Szent-Györgyi's original citrin (Lavollay *et al.*, 1943; Parrot *et al.*, 1944), and the glycoside of esculetol (6,7-dihydroxycoumarin) is equally active (Lavollay and Sevestre, 1944). Employing a guinea pig capillary resistance test, a response could be elicited with as little as 1 γ of either of the two compounds. A review covering these and other vitamin P substances has been published recently (Javillier and Lavollay, 1946).

In view of these considerations it is impossible to speak accurately of a vitamin P *requirement.* Further elucidation of the chemical nature and physiological properties of vitamin P must be forthcoming before its requirement by the guinea pig can be discussed in the conventional manner.

IX. ANTISTIFFNESS FACTOR

The guinea pig wrist stiffness syndrome was first observed by Wulzen and Bahrs (1941) and has been studied in considerable detail by the Oregon group (van Wagtendonk *et al.*, 1943, 1944a, b, c, d, 1945a, b, 1946a, b; Simonsen and van Wagtendonk, 1947; Bahrs and Wulzen, 1947; Lansbury *et al.*, 1947; Weimar and Wulzen, 1948; Christensen *et al.*, 1948). Guinea pigs raised on pasteurized skimmed milk or semipurified yeast diets developed a stiffness of the wrists which could be cured by the daily feeding of 1 cc. of raw cream or 0.1 γ of a highly purified concentrate obtained from cream (van Wagtendonk and Wulzen, 1943). As the deficiency progressed the muscles were found to be atrophied and streaked with calcium. Calcium deposits were found under the skin, in the joint regions, between the ribs and indiscriminantly in many organs including the heart and aorta. The fat-soluble, heat-labile principle in cream responsible for the reversal of the early stiffness condition was named the "antistiffness factor."

At one time methyl vinyl ketone was thought to be the active ingredient in cream (Wulzen and Bahrs, 1941) but this belief was later abandoned (van Wagtendonk and Wulzen, 1943). A crystalline material has now been isolated from sugar cane juice which cures the stiffness syndrome in doses of 0.002 γ fed daily for 5 days (van Wagtendonk and Wulzen, 1946b). If this seems an incredibly small dose, consider the suggestion of Simonsen and van Wagtendonk (1947) that the activity of the isolated crystalline material may be due to traces of the active ingredient!

Confirmation of these findings in other laboratories has been slow in materializing (Homburger and Reed, 1945; Kon *et al.*, 1946), but recently at least partial substantiation of the Oregon work has been obtained (Anderson and Caldwell, 1946; Hogan and Regan, 1946; Oleson *et al.*, 1947; Mannering *et al.*, 1948). Oleson and coworkers (1947) observed the stiffness syndrome in guinea pigs receiving a commercial stock ration and found over 24 known steriod materials possessing antistiffness activity. Among the more active compounds were ergostanol and several of its esters which produced responses in daily doses of from 1 to 5 γ. The 500-fold discrepancy in potency between the most active compounds of Oleson *et al.* and the crystalline material of van Wagtendonk and Wulzen would more than suggest a lack of identity of the substances. However, there is little justification in making such quantitative comparisons. The Oregon workers (Christensen *et al.*, 1948) have treated their data statistically and have concluded that "the assay procedure is not suitable for quantitative analytical purposes."

The observed derangement in calcium and phosphorus metabolism in deficient animals (van Wagtendonk *et al.*, 1944a, b, 1945a) and the fact that wrist stiffness has not been noted in guinea pigs receiving diets of higher purity than those used by the Oregon workers raise the question as to whether the stiffness syndrome is a true nutritional deficiency or a pathological condition arising from excesses of calcium and phosphorus, or the imbalance of these elements in the special diets employed. This aspect of the problem certainly warrants more careful scrutiny than it has received.

The antistiffness problem has been reviewed critically in a recent publication (Anonymous, 1948).

X. UNIDENTIFIED FACTORS

There is general agreement among all who have investigated the problem that the crystalline vitamins do not satisfy all of the vitamin requirements of the guinea pig. Agreement is not unanimous, however, as to the number and kind of new factors required. Table II lists these postulated factors. This list is not intended to include all of the many instances in the literature where attempts to raise guinea pigs on experimental diets have failed, but contains those postulated factors which have been more specifically defined and which have not as yet been canceled by subsequent identification of the factor.

TABLE II

Unidentified Factors Postulated for the Guinea Pig

Name or source	Author
Factor J	Euler *et al.*, 1935
Grass juice factor	Elvehjem *et al.*, 1934; Kohler *et al.*, 1938
Lettuce, grass	Cannon and Emerson, 1939
Yeast	Hogan and Hamilton, 1942
GPF-1	Woolley, 1942
GPF-2	Woolley, 1942
GPF-3	Woolley, 1942
Commercial casein	Kuiken *et al.*, 1944
Rice polish concentrate, yeast, etc.	Kuiken *et al.*, 1944

Factor J was described by Euler *et al.* (1935) as a material in fruit, especially St. John's berries, which protects guinea pigs against pneumococal infections. Ascorbic acid was found to be less effective for this purpose than lemon juice and it was therefore concluded that a special antipneumonia factor exists. As further differentiation from ascorbic acid, factor J does not occur in paprika or in the eye lens.

While studying the effect of seasonal variation on the nutritive value of milk, the Wisconsin group (Elvehjem *et al.*, 1934; Stirn *et al.*, 1935) noted that milk from cows fed a winter diet was inferior to milk from cows kept on summer pasture. Supplementing "winter milk" with concentrates of vitamins known to exist at the time did not improve the growth of rats. The term "grass juice factor" was applied to the substance or substances in "summer milk" and in green plants responsible for the stimulation of rat growth (Kohler *et al.*, 1937). The rat proved to be a rather unsatisfactory subject for the assay of the grass juice factor. While attempting to improve the assay, Kohler *et al.* (1938) reasoned that a strict herbivore might be more sensitive to the deficiency since such animals normally subsist on fresh green foods. This was the case. The grass juice factor was described as being water soluble, labile to heat and oxidation, and adsorbable on Norit (Kohler *et al.*, 1939b; Randle *et al.*, 1940).

Cannon and Emerson (1939) were unable to obtain proper development of guinea pigs fed a purified ration known to be adequate for the rat. The addition of lettuce, grass, or grass extracts greatly improved growth. They believed their factor to be the grass juice factor.

Because "winter milk" is not regularly obtainable in most laboratories, Kohler *et al.* (1939a) devised a semipurified yeast ration to replace milk. Although the existance of the grass juice factor could be demonstrated using this diet, the common occurance of stomach and intestinal ulcers interfered with the assay. Ground whole oats prevented the ulcers but were not very effective in promoting growth. Cholic acid and chondroitin, which prevent gizzard erosion in the chick (Almquist and Mecchi, 1938; Bird *et al.*, 1938), were ineffective in protecting guinea pigs against ulcers. The authors decided that the ration was lacking in at least two factors, the grass juice factor, and a factor associated with the prevention of ulcers. Milk was considered a good source of the stomach ulcer factor (Sober, 1942). The Wisconsin workers (Cannon *et al.*, 1945) have stated their belief that these stomach and intestinal lesions may be due to salmonella infection. This does not necessarily preclude the concept of a nutritional etiology of the ulcers. Watson (1937) showed that the feeding of dried skimmed milk increased the resistance of mice to deliberate infection with *S. aertrycke* (*Bact. typhi-murium*). The relationship of diet to resistance to infection with salmonella has received careful study by Schneider and Webster (reviewed by Schneider, 1946).

Continuing Kohler's investigations, Sober *et al.* (1942) found that lesser amounts of sources of the grass juice factor could be employed in a purified diet if certain other crude materials were fed. This led to the postulation of three guinea pig factors, one in grass, a second in yeast, and a third in milk. This is at variance with the results of Hogan and Hamilton (1942), who maintain that yeast supplies all of the unidentified vitamins required by the guinea pig. However, the latter workers employed cellulose in their diet and this was found to be responsible for the different results obtained (Sober *et al.*, 1942).

Wooley (1942) has presented evidence for the existence of three dietary essentials for the guinea pig. These factors were designated GPF-1, present in a 50% ethanol extract of linseed oil meal, GPF-2, a factor remaining in the alcohol extracted linseed oil meal, and GPF-3, a third factor postulated on the basis that the addition of linseed oil meal alone to a purified ration would not support growth or survival. The Wisconsin workers (Mannering *et al.*, 1943) were unable to identify two factors in linseed oil meal but confirmed the observation that linseed oil meal contributes greatly to the basal ration. Unlike Wooley, who was unable to find any good sources of GPF-3, they demonstrated a fairly wide distribution of this factor. Liver is a particularly good source. Hence, while adhering to the terminology proposed by Woolley, the Wisconsin investigators were not convinced that what they called GPF-3 was identical with the GPF-3 of Woolley. They described GPF-3 as being water soluble, stable to autoclaving under alkaline conditions but labile to acid autoclaving, and poorly adsorbed on Norit. GPF-3 was not thought to be folic acid.

Later, Woolley and Sprince (1944, 1945) found that when 20% of cellulose was included in the diet and the casein content increased from 20% to 30%, the linseed oil meal factor, GPF-2, was no longer required by the guinea pig. It was further decided that GPF-1 is identical with folic acid. This last conclusion is hardly tenable in view of the low folic acid content of linseed oil meal, the material from which GPF-1 was originally obtained. A folic acid deficiency can be produced in guinea pigs fed a diet containing 25% of linseed oil meal (Mannering and Brown, 1946).

Crampton and Bell (1947b) have also found cellulose to be a valuable addition to the diet of the guinea pig. Yet cellulose does not appear to be essential. Using a milk diet, Kohler *et al.* (1938) obtained good growth of guinea pigs without feeding cellulose or other types of roughage. It is well established that certain nonvitamin constituents of the diet, particularly carbohydrates, greatly reduce the vitamin B requirements of rats (reviewed by Najjar and Barrett, 1945). By serving as media for intestinal microorganisms these materials are thought to increase the number of bacteria in the lower tract or to alter the flora to one capable of greater vitamin synthesis. Cellulose may function in this manner in the guinea pig. It may also act to delay the passage of food through the upper tract thereby permitting more efficient utilization of vitamins that may be limiting in the diet. Material is known to pass very rapidly through the upper tract of the guinea pig before reaching the cecum (Reid and White, 1948).

Kuiken and coworkers (1944) concluded from their studies that at least two unidentified factors are required to complete the diet of the guinea pig. When commercial casein was used in the diet, small supplements of rice polish concentrate, brewers' yeast, liver extract, skimmed milk powder or dried grass markedly improved both growth and survival. If purified casein was used, these supplements, even in large amounts, had little effect, thereby providing evidence for another factor in commercial casein.

The meager information available describing the properties of these postulated factors does not permit generalizations as to which are identical. Much of this work should be repeated now that crystalline folic acid is available.

XI. Normal Growth of Young Guinea Pigs

In the literature dealing with guinea pig nutrition one encounters such adjectives as "normal" or "excellent" used in reference to the growth of animals responding to treatment or serving as positive controls. "Normal" growth is usually defined as the average growth obtained by animals receiving the stock ration used in that laboratory where the investigation is being conducted. Too often this does not represent the guinea pigs' full capacity for growth. Experience with the rat has shown that as nutritional knowledge has progressed, values for "normal" growth have altered, always in an ascending direction, until a more or less acceptable maximum has been reached now that the picture of rat nutrition is approaching completeness. Knowledge of guinea pig nutrition is so incomplete that it is unwise at this time to accept an absolute value for maximum growth. Nevertheless, the best growth obtained to date should serve as a measure of normalcy until a still higher value is reported. Failure to recognize the full capacity of the guinea pig for growth has resulted in misinterpretation of data. An example of this has been pointed out in the discussion of the ascorbic acid requirement of the guinea pig for growth.

Table III lists the growth responses of young guinea pigs reported by investigators using crude diets. The discrepancy between the poorest and best growth obtained is almost twofold. Maximum growth is rarely achieved with experimental diets. Appetite is often thought to play an important role when unnatural diets are being fed to guinea pigs, and their failure to promote optimal growth responses has been attributed to the guinea pigs' unwillingness to accept the rations wholeheartedly. In this connection it is remembered that in the past this same explanation was offered when rats did not respond maximally to purified diets. The perfection of purified diets for the rat containing adequate numbers and amounts of the vitamins has made it less frequently necessary to resort to such explanations.

TABLE III

Growth of Guinea Pigs Fed Stock Diets

Author	Daily gain[1] (g./day)
Minot, 1891	4.3
Paton and Goodall, 1904	3.9
Bessesen and Carlson, 1922	5.2
Glimstedt, 1936	3.6[2] 7.2[3]
Hogan and Hamilton, 1942	4.2[4]
Woolley, 1942	5.7[5]
Cannon *et al.*, 1945	7.5

[1] Based on growth from second to eighth week of life unless otherwise indicated.
[2] Winter diet.
[3] Summer diet.
[4] Based on growth from fourth to tenth week of life.
[5] Based on growth from first to fourth week of life.

XII. VITAMIN CONTENT OF EXPERIMENTAL DIETS CURRENTLY USED

To rule out as many nutritional variables as possible it is sought in many studies only to supply an abundant intake of the crystalline vitamins without regard to the actual requirements. To accomplish this, investigators have had to refer to the requirements of other animals in formulating their experimental diets for guinea pigs. The vitamin contents of four diets which have been used recently in studying unidentified factors are tabulated in Table IV. The levels of vitamin E in some of these diets do not provide a margin of safety. At the time three of the diets were devised, pteroylglutamic acid was not available. Recent studies point to the advisability of adding this newer member of the vitamin B complex to the list. Based on rat requirements, all of the other vitamins are provided in superabundance.

XIII. SUMMARY

Any review of the nutrition of the guinea pig at this time must emphasize the lack of knowledge of the vitamin requirements of this species. Acute deficiencies of vitamins A and E have been repeatedly demonstrated without establishing quantitative requirements. An examination of the literature reveals meager or inconclusive evidence for the production of thiamine, riboflavin, pantothenic acid, pteroylglutamic acid, biotin, and vitamin K avitaminosis, while no attempts appear to have been made to determine even the qualitative pyridoxine or choline requirements. Only the requirement for ascorbic acid has been determined accurately. An explanation for this lack of information has been given.

TABLE IV

Vitamin Content of Experimental Diets Currently Used[1]

	Diets			
	I[2]	II[3]	III[4]	IV[5]
Ascorbic acid, mg. (mg./day)	(10)	10	(1–20)	(20)
Vitamin A, I.U. (I.U./week)	[6]	2000	1200	(5,500)
Vitamin D, I.U. (I.U./week)	[6]	100	170	(500)
Vitamin E, mg. (mg./week)	(1)	4	2–4	(7.5)
Thiamine hydrochloride, mg.	0.25	0.2	0.8	0.4
Riboflavin, mg.	0.35	0.5	1.6	0.5
Pyridoxine hydrochloride, mg.	0.25	0.2	1.2	0.4
Ca pantothenate, mg.	1	1	2	1.5
Niacin, mg.	5	1	2	2
Biotin (γ/day)				(4)
Pteroylglutamic acid, mg.				2
Choline chloride, mg.	400	100	300	100
Inositol, mg.		100	50	100
PABA, mg.			20	25
2-methyl-1,4-naphthoquinone, mg.		0.1	2	2.5

[1] Per 100 g. of ration unless otherwise indicated in parentheses.
[2] Sober *et al.*, 1942.
[3] Woolley, 1942.
[4] Hogan and Hamilton, 1942.
[5] Mannering and Brown, 1946.
[6] Four drops of Haliver oil/week.

Using the rat for comparison, as is the general practice, several peculiarities have been noted in the vitamin nutrition of the guinea pig. The inability of the guinea pig to synthesize ascorbic acid is the best-known example. The guinea pig is much less able to convert carotene or to store vitamin A than the rat. The guinea pig is more susceptible to a dietary lack of vitamin E, showing signs of muscle dystrophy very early in the course of deficiency. The feeding of excessive amounts of niacinamide does not deplete the guinea pig of labile methyl reserves as it does the rat. The wrist stiffness syndrome resulting from a lack of the anti-stiffness factor has not been observed in the rat. This may reflect a fundamental difference in the calcium and phosphorus metabolism of the two species. Purified diets which support the development of rats, when fed to guinea pigs, result in failure of growth and survival; still unidentified factors are required by the guinea pig.

The review affirms the need of further study of the nutrition of this useful laboratory animal.

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The Chemistry of Gonadotropic Hormones

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I. INTRODUCTION

Gonadotropic hormones may be divided into two main groups, one of which is derived from pituitary and the other from living chorionic tissue. The former includes those obtained directly from the extracts of pituitaries and from the blood and urine of normal men and women and of women at menopause. The second group of gonadotropins consists of the active material of the urine or blood of women during pregnancy and of the blood of pregnant mares. Biological studies* of gonadotropic hormone reveal certain interesting characteristics which may be taken to classify them into three classes: one is concerned chiefly with the follicle or the seminiferous tubule growth, the second with the process of luteinization or with the stimulation of interstitial cells, and the last class deals with both of those two types of reactions. For instance, the active principle in the urine of menopausal women produces only follicular stimulation, while the gonadotropin of pregnant women's urine or human chorionic gonadotropin (HCG) causes reactions ascribed to the interstitial cell-stimulating hormone (ICSH) of the pituitary gland. On the other hand, the gonadotropin of pregnant mare's serum (PMSG) evokes physiologic responses similar to the combined effects of those produced by the gonadotropic principles of human menopausal and human pregnancy urine.

Within the past decade considerable progress has been made toward the purification and isolation of the gonadotropic hormones. In 1939, Gurin *et al.* described a method for the preparation of a highly purified gonadotropic hormone from human pregnancy urine. Recently Claesson *et al.* (1948) claimed that the hormone was isolated in crystalline form. Procedure for isolation of interstitial cell-stimulating hormone from either sheep or pig pituitaries has already been reported (Li *et al.*, 1940b, c; Shedlovsky *et al.*, 1940). The isolation of the follicle-stimulating hormone (FSH) from sheep glands has very recently been described by Li *et al.* (1949). Highly purified preparations of pregnant mare serum gonadotropin were obtained by various investigators (Goss and Cole, 1940; Li

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* For the physiological function of the gonadotropins, see Fevold (1944) and Levin (1944).

et al., 1940a; Rimington and Rowlands, 1944) though this hormone has not been isolated in pure form.

The gonadotropic hormones are proteins. In the purification of these hormones, it is important to know the purity of the final product. Methods generally employed in defining the purity of a simple chemical compound cannot be applied to proteins. In order to define how pure a protein is, we have to rely on certain special properties of the molecule. These properties must be derived from the molecular characteristics which may be of a physicochemical or biological nature. If one finds that a protein possesses uniformity of these properties, it is highly probable that the protein is a pure substance. The few properties which provide satisfactory criteria* of purity are ultracentrifugation, diffusion, electrophoresis, solubility, and biological activity. Among these tests, solubility is considered to be the best criterion for the purity of a protein, because it is based on the theory of phase rule, as the melting point is employed for purity test in organic chemistry.

It should not be overlooked that success in isolating biologically active principles from natural sources is markedly dependent on the sensitivity and accuracy of bioassay. Thayer (1946) and Li and Evans (1948) have recently reviewed various methods for the biological assays of the gonadotropins. It is sufficient to say here that should specific and accurate assay results be desired, we must employ hypophysectomized animals, for the interference of the animal's own hypophysis often gives rise to errorous conclusions.

A few words may be said regarding the terminology of the gonadotropic hormones. The three gonadotropic hormones in pituitary tissues were named according to their physiological actions: follicle-stimulating hormone (FSH), luteinizing or interstitial-cell stimulating hormone (LH, ICSH) and lactogenic hormone (prolactin, luteotropin). Although it was found later that FSH also stimulated spermatogenesis in the male, no other name has been proposed other than that suggested by Coffin and van Dyke (1940) who proposed a Greek name, thylakentrin, for FSH.

At one time there was some confusion as to the identity of the interstitial-cell stimulating and the lutinizing factors. It was subsequently shown that luteinizing hormone (LH) produced interstitial cell hypertrophy in both male and female gonads. Since LH implies only the action in female, we feel that the name ICSH (interstitial cell-stimulating hormone) should be employed to signify the luteinizing factor of the pituitary. Coffin and van Dyke (1940) also proposed a Greek name, metakentrin, for ICSH.

* It is now well recognized that crystallinity is a poor criterion of purity. For a detailed discussion on the criteria of the purity of a protein, see Li (1949).

The inclusion of lactogenic hormone as one of the pituitary gonadotropins was induced from the work of Evans *et al.* (1941) who found that pure lactogenic hormone can stimulate or intensify corpus luteum function. Hence, the name, luteotropin, has been proposed and employed by various investigators. It should be emphasized that the terms lactogenic hormone, prolactin and luteotropin are synonymous and refer to the same substance. The physiology and chemistry of this substance are discussed elsewhere in this volume.

The gonadotropins in serum or urine were given names which denoted their sources. For instance, the hormone occurring in the serum of pregnant mares is called pregnant mare serum gonadotropin (PMSG) and that in the urine of pregnant women the human chorionic gonadotropin (HCG). The term, PU, has sometimes been employed to represent HCG (Levin, 1944). Cole *et al.* (1940) suggested the term "equine gonadotropin" for PMSG.

II. Anterior Pituitary Gonadotropins

1. General

In 1927 Smith clearly showed that the pituitary was essential for gonadal development and concluded that gonadotropins existed in the anterior pituitary. Four years later Fevold *et al.* (1931) obtained two components from certain pituitary gonadotropic extracts: one concerns mainly the stimulation of follicle growth in the ovaries and increase of spermatogenic activity in the male, and the other the transformation of follicles to corpora lutea in the ovaries and the enlargement of the secondary sex glands in the male. The former is called follicle-stimulating hormone (FSH) and the latter interstitial cell-stimulating hormone (ICSH). Subsequent works from other laboratories confirmed these findings of Fevold *et al.* It is now established beyond any doubt that two hormones (FSH and ICSH) are present in the pituitary extract and are responsible for the gonadotropic activity.

In 1940, Li *et al.* 1940b, c and Shedlovsky *et al.* independently described a method for the isolation of ICSH in pure form from sheep and pig glands, respectively. Both preparations behaved as a single protein in electrophoresis, ultracentrifuge, and solubility studies. It is especially important that they are freed from contamination of the other gonadotropic hormone, FSH. It will be seen that the same hormones isolated from sheep or pig pituitary are not identical substances although their biological characteristics are very similar.

Within the last two decades, highly purified FSH preparations have been reported by various investigators (Li and Evans, 1948). The isolation of this hormone in pure form has been achieved only recently (Li, 1949; Li *et al.*, 1949). FSH is particularly distinct from other known hormones of the anterior pituitary in that it is the only one which can not be precipitated by half saturation of ammonium sulfate.

As already mentioned in the Introduction, lactogenic hormone is now known as the third gonadotropin of the hypophysis, the chemistry of which is not discussed in this review; the reader may refer to the article by White and Dougherty in this volume. The gonadotropic action of lactogenic hormone is to initiate and maintain the secretory activity of the functional corpora lutea. Although it is outside the scope of this review to discuss the biological properties of these pituitary gonadotropins, it should be pointed out that the complete function of an ovary, for instance, depends upon the interplay of FSH, ICSH, and lactogenic hormone. This may be illustrated in Fig. 1. Since ICSH has already been isolated in pure form for some time and since FSH has only recently been prepared in highly purified state, it is natural that our knowledge of the biological functions of ICHS surpasses that of FSH. In fact, little is known about the physiological function of FSH except that it promotes follicular growth in both normal and hypophysectomized immature animals. It may be well at this time to summarize the known biological properties of ICSH (Li *et al.*, 1940b; Simpson *et al.*, 1942; Greep *et al.*, 1942; Simpson *et al.*, 1944) as follows: (a) repair of the ovarian interstitial tissue of the immature hypophysectomized rats; (b) stimulation of the

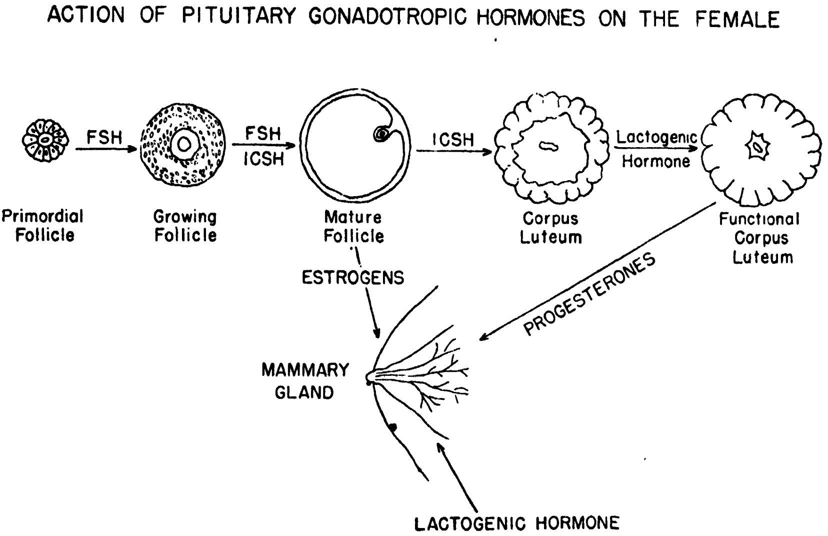

FIG. 1. Action of pituitary gonadotropic hormones on the female.

seminiferous tubules and the interstitial cells in the male, causing androgen production to enlarge the accessory organs; (c) antagonism against chorionic and pregnant mare serum gonadotropin when injected intraperitoneally in normal immature female rats but not in hypophysectomized animals; (d) formation of corpora lutea and production of estrogen in immature hypophysectomized rats when injected subcutaneously in conjunction with FSH; (e) production of ovulation in normal rabbits; (f) prolongation of diestrous interval in adult normal rats; (g) formation of placentoma in normal but never in hypophysectomized rats; (h) prolongation of gestation in normal rats, and (i) maintenance of spermatogenesis if given immediately after hypophysectomy.

2. *Interstitial Cell-Stimulating Hormone (ICSH)*

a. Occurrence. Fevold (1943) has summarized the content of ICSH in the pituitaries of various animals (Table I). Sheep glands have higher ICSH content than that of pig, while beef and horse pituitaries are low in interstitial cell stimulating activity. There are some indications that ICSH may be found in small amounts in the urine of human subjects throughout life. It has also been reported detectable in the human blood.

TABLE I

Interstitial-Cell Stimulating Hormone Content of the Pituitaries of Various Species

High	Moderate	Low
Cat	Rat	Beef
Baboon	Armadillo	Whale
Sheep	Guinea Pig	Horse
Opossum	Dog	Human
Cottontail Rabbit	Domestic Rabbit	

(From Fevold, H. L. *Ann. N. Y. Acad. Sci.* **43**, 324, 1943.)

For the isolation of ICSH, pituitary glands of sheep or pigs are commonly used. These glands, as secured from slaughter house, may be stored immediately in frozen state. We have found that the ICSH activity remained unchanged as long as the glands are kept below −5°C. They may sometimes be desiccated with acetone or by lyophilization and kept as dry powders.

b. Method of Isolation of the Sheep Hormone. The method of Li *et al.* (1940b, 1940c, 1942) for the isolation of ICSH from acetone-dried sheep glands as a homogeneous protein is given below; all steps were carried out in a cold room at 4°C.

1. The 40% alcohol extract of acetone-dried sheep pituitary was used as the starting material. The extract was precipitated with alcohol and dried with absolute alcohol and ether.

2. Fifty grams of this powder was extracted with 3 liters of water; the extract was adjusted to pH 4.5 and was precipitated with an addition of equal volume of cold acetone.
3. The precipitate was extracted with 1% NaCl solution. The extract was brought to 0.5 saturation with SAS.* The precipitate obtained was reprecipitated twice more under similar conditions.
4. The third 0.5 SAS precipitate was dissolved in water and dialyzed until salt-free. The dialyzed solution was brought to 0.2 saturation with SAS. After the removal of 0.2 SAS precipitate, the supernatant was brought to 0.4 SAS. This procedure was repeated twice more.
5. The final 0.2–0.4 SAS precipitate was dissolved in water and dialyzed. The dialyzed solution was brought to 0.33 saturation with SAS and the precipitate formed was centrifuged off. The clear supernatant was adjusted to pH 4.1. This isoelectric precipitation may be repeated one or two more times and the final precipitate represents the pure ICSH.

From 1 kg. of fresh sheep pituitary approximately 0.1 g. of ICSH may be obtained by this procedure. It may be noted in Table II that the interstitial cell-stimulating activity of the final product is more than 1000 times higher than the starting material.

TABLE II

Yield and Potency of ICSH Fractions from Sheep Pituitary

Fraction	Weight	ICSH[1]	FSH[2]	Ratio
	g.	R.U./g.	R.U./g.	
Frozen fresh gland	4,000.0	110	67	1.7
Acetone dried gland	1,200.0	1,000	400	2.5
40% Alcohol powder	50.0	20,000	4,000	5.0
0.5 SAS precipitate	7.0	20,000	2,000	10.0
Purified ICSH	0.5	100,000–200,000	350	300–600

[1] R.U. ICSH is the minimum dose administered intraperitoneally (conditions as FSH assay) which causes interstitial tissue repair.

[2] One R.U. of FSH is the minimal amount which, given subcutaneously in 3 daily doses, followed by autopsy 72 hour after first injection, causes resorption of follicular growth in hypophysectomized female rats (26–28 days at operation, 6 to 8 days postoperative onset of injections).

(From Li, C. H., Simpson, M. E., and Evans, H. M. *Endocrinology* **27**, 804, 1940.)

c. Method of Isolation of the Pig Hormone. Using fresh pig pituitary as their original material, Shedlovsky, Chow and their collaborators (1940, 1942) obtained a pure ICSH preparation in the following manner.

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*SAS will be used throughout as an abbreviation for saturated ammonium sulfate.

1. The ground fresh pig glands were extracted with 2% NaCl at 4°C. and the extract was adjusted to pH 4.2–4.6.
2. The supernatant was saturated with ammonium sulfate; the precipitate formed dissolved in water and dialyzed. The dialyzed solution was next adjusted to pH 5.1–5.5.
3. The supernatant was brought to 0.33 saturation with ammonium sulfate. The 0.33 SAS soluble fraction was brought to 0.90 saturation with further addition of the salt.
4. The 0.33–0.90 SAS precipitate dissolved in water and dialyzed; ammonium sulfate was added to the dialyzed solution until 0.33 saturation. After centrifuging off the precipitate formed, the supernatant was adjusted to pH 7.3.
5. The isoelectric precipitation was repeated at least seven times. The final pH 7.3 precipitate at 0.33 SAS solution is the pure hormone.

The yield of the hormone according to this procedure was 0.92 g. of N from 4.53 kg. of fresh gland.

TABLE III

Physicochemical Characteristics of Sheep and Pig Interstitial Cell-Stimulating Hormones

Determinations	Sheep	Pig
N, %	14.20	14.93
Molecular weight	40,000	100,000
Isoelectric point, pH	4.6	7.45
Sedimentation constant, S	3.6	5.4
Tyrosine, %	4.5	
Tryptophan, %	1.0	3.8
Mannose, %	4.5	2.8
Hexosamine, %	5.8	2.2

d. Are Sheep and Pig ICSH Identical? Table III summarizes the physicochemical characteristics of ICSH isolated either from sheep pituitaries or pig glands. It is evident that the two preparations are quite different chemically. Among the known properties, the differences in molecular weight and isoelectric point are most marked; the molecular weight of sheep hormone is 40,000 and that of pig 100,000, while the isoelectric point of sheep ICSH is at pH 4.6 and that of pig at pH 7.45.

The fact that sheep and pig ICSH are not the same chemical entity is further shown by immunological methods. Chow (1942) was able to show that pig ICSH can readily be distinguished immunologically from the sheep hormone. It was found that pig ICSH stimulated the production of specific antibodies in rabbits and that the antiserum formed did not react with sheep ICSH in the precipitin and complement fixation reactions.

Some differences in biological potency of these two hormones have also been reported. Greep *et al.* (1942) have found that the sheep ICSH is far more active in the repair of the ovarian interstitial cells in hypophysectomized rats and in causing ovulation in rabbits than is the pig hormone. However, the two hormones are equally effective in stimulating the anterior prostate.

e. Effect of Various Agents on ICSH Activity. Crystalline chymotrypsin, trypsin and pepsin destroyed the gonadotropic action of ICSH (Chow *et al.*, 1939). Picrolonic, flavianic, picric, and trichloroacetic acids precipitated ICSH completely with retention of its physiological activity (Fevold, 1939; Li *et al.*, 1940b). The reactions of ketene and cysteine with ICSH caused a complete destruction of the hormonal potency indicating that free amino and disulfide groups may be essential for the biological activity of ICSH (Li *et al.*, 1940b; Fraenkel-Conrat *et al.*, 1939).

3. *Follicle-Stimulating Hormone (FSH)*

a. Method of Isolation. Human, horse, sheep, and pig pituitaries are known to contain high concentration of FSH. The extraction of FSH from these pituitary tissues is generally made either with saline or alcoholic solutions like those employed for the isolation of ICSH. There are methods which enable one to obtain highly purified preparations. Fevold *et al.* (1940) employed frozen sheep glands as their starting material and extracted with dilute aqueous ammonium hydroxide at pH 8.0; by fractionation with ammonium sulfate they found that FSH is soluble at 2.4 *M* but precipitated at 2.7 *M* ammonium sulfate. Fraenkel-Conrat *et al.* (1940) used 40% alcohol extract of acetone-dried sheep glands and precipitated FSH out from the extract in 0.50–0.67 SAS solution. McShan and Meyer (1940) extracted acetone-dried sheep pituitary with water and digested the extract with crude trypsin; it was found that FSH was resistant to the enzyme action and thus could be recovered from the enzyme digest by alcohol precipitation. Greep *et al.* (1940) have described a method to obtain a biologically pure FSH from pig glands, which is based on the fact that the hormone is soluble in a pH 4.4 acetate buffer containing 20.5% sodium sulfate whereas ICSH is insoluble in this solvent. This preparation of Greep *et al.* was shown to be heterogeneous in ultracentrifugal and electrophoretic experiments (Chow, 1943).

Success in the isolation of FSH in pure form was only recently achieved by Li *et al.* (1949); their method, carried out at 2–3°C., may be described as follows:

1. *Ammonium sulfate fractionation.* One kilogram of frozen sheep pituitary is finely ground and extracted with $Ca(OH)_2$ according to the procedure described previously (Li *et al.*). After the removal of the precipitate obtained by adding saturated $(NH_4)_2SO_4$ (SAS) to half saturation, the supernatant was brought to 0.75 saturation by addition of solid $(NH_4)_2SO_4$. This precipitate was dissolved in water and dialyzed. A slight precipitate that formed during dialysis was discarded. The clear reddish supernatant solution was adjusted to pH 6.0 and then to pH 4.7. The precipitate formed at these two pH regions was removed by centrifugation. The supernatant was brought to 0.5 SAS by slow addition of an equal volume of SAS solution at pH 4.7. The precipitate formed was removed and the supernatant again brought to 0.75 SAS by further addition of SAS solution at the same pH. The precipitate was dissolved and dialyzed. The whole procedure was repeated once more; the final dialyzed solution was frozen and dried in vacuum. The product is called "crude FSH" and may be kept in a dessicator for further use.

TABLE IV

Yield and Potency of FSH Fractions Obtained in the Purification Procedure from Sheep Glands

Fraction	Weight	FSH*
	g.	R.U./g.
Fresh sheep gland	1,000.0	67
"Crude FSH"	3.0	2500
40% Ethanol extract	1.2	6700
Purified FSH	0.5	20,000

* One R.U. of FSH is the minimal amount which, given subcutaneously in 3 daily doses, followed by autopsy 72 hours after first injection, causes resorption of follicular growth in hypophysectomized female rats (26–28 days at operation, 6 to 8 days postoperative onset of injections).

2. *Alcohol fractionation:* The "crude FSH" powder was next extracted with 0.10 *M* K_2HPO_4 in 40% alcohol. After the removal of the residue, the alcohol concentration in the supernatant was increased to 80% by slowly adding cold 95% alcohol (−5°C.). The precipitate was dissolved in water and dialyzed.

3. *Further ammonium sulfate fractionation:* The dialyzed solution was adjusted to pH 4.7 and centrifuged if precipitation occurred. A pH 4.7 SAS solution was next added until the concentration became 0.55 saturation. The 0.55 SAS precipitate, found to be devoid of FSH activity, was removed by centrifugation. The supernatant was brought to 0.70 saturation with more pH 4.7 SAS solution; the precipitate formed was dissolved in water and dialyzed. This 0.55–0.70 SAS fractionation was repeated twice.

The final 0.55–0.70 SAS precipitate is the pure FSH. It behaves as a single protein in electrophoresis and ultracentrifuge studies. Approximately 0.5 g. FSH may be obtained from 1 kg. fresh sheep glands. Table IV presents a summary of the yield and potency of the fractions obtained in each step of purification from more than twenty experiments. All FSH activity was tested on female rats hypophysectomized at 27 days of age. Subcutaneous injections were begun about 7 days later and were given once daily for 3 days. Autopsy was performed 72 hours after the onset of the injections. Histological examination of the ovaries showed that a total dose of 0.05 mg. of the preparation initiated follicular development, i.e., an increase in follicle size beyond that characteristic of controls and beginning antrum development.

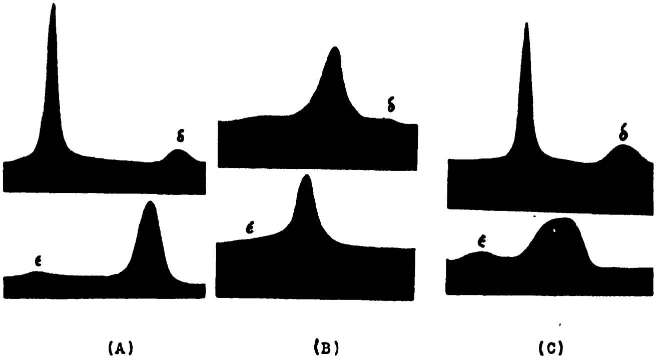

FIG. 2. Electrophoresis patterns of follicle-stimulating hormone solutions in buffers of ionic strength 0.1 at 2°C. (A) pH 7.0 phosphate buffer; 1.2% solution; 3 hours electrolysis at 6.2 volts per cm. (B) pH 5.1 acetate buffer; 0.65% solution; 4.5 hours electrolysis at 6.3 volts per cm. (C) pH 4.0 acetate buffer; 1.2% solution; 4 hours electrolysis at 5.8 volts per cm.

b. Criteria of Purity. The hormone isolated by the procedure just described has been tested for its purity by biological, electrophoretic and ultracentrifugal experiments. The solubility test has not been applied and thus it cannot be said that the preparation is absolutely pure.

1. *Biological test.* One of the difficulties in purifying FSH is its freedom from the other gonadotropic component, ICSH. For instance, the highly purified preparation of Fraenkel-Conrat *et al.* (1940) showed ICSH contamination at ten times its minimum effective dose (MED) and estrous uteri at four times MED. When a total dose of 2.0 mg. of our purified product, administered over a 4-day period in hypophysectomized female rats, did not show interstitial-cell stimulating, thyrotropic, adrenocorticotropic, or growth-promoting activities it indicates that the preparation was substan-

tially free of other active components. It was also repeatedly found that three hundred times the MED (300 × 0.05 = 3.0 mg.) did not show estrous uteri in hypophysectomized rats whether it was administered subcutaneously or intraperitoneally.

2. *Electrophoresis.* When 1.0–1.5% solutions of the preparation were subjected to a conventional Tiselius apparatus with the Longsworth scanning method, in every case a single boundary appeared and no indications of the presence of other contaminating materials. In these experiments, six different buffers of ionic strength 0.10 at different pH's were employed and the electric current was applied more than 4 hours in a potential gradient of approximately 6 volts per cm. It is evident from Fig. 2 that the scanning patterns of the ascending boundaries of FSH solutions in 4 different pH's behave as a single protein.

In one experiment, the protein which appeared in three different sections of the electrophoresis cell after it had been electrolyzed for 4 hours at pH 7.0 was recovered separately. The assay results of the three fractions indicated that no significant difference in follicle-stimulating activity could be detected. Since no electrophoretic separation of the hormone could be achieved, the preparation may be said to be homogeneous as to its electrochemical properties.

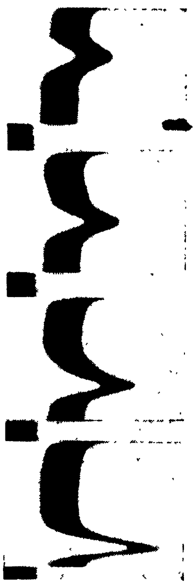

Fig. 3. Four schlieren patterns of a saline solution of the pituitary follicle-stimulating hormone taken at 1920-seconds intervals during sedimentation at 165,000 times gravity in an ultracentrifuge.

3. *Ultracentrifuge.* One experiment was carried out in a Spinco ultracentrifuge with a hormone concentration of 0.5% in saline solution. The sedimentation velocity was at 165,000 times gravity. The results recorded in Fig. 3 indicate that the preparation is essentially monodispersed.

c. Physicochemical Characteristics. The results of analysis of one specimen of the pure hormone were: carbon, 44.93%; hydrogen, 6.67%; nitrogen, 15.10%. Organic sulfur was detected and accurate estimation of the sulfur content is yet to be determined.

The presence of hexose and hexosamine in highly purified FSH has been known for some time. Gurin (1942) reported that the pig preparation of Greep *et al.* (1940) contains 4.5% mannose and 44% hexosamine. Evans, Fraenkel-Conrat *et al.* (1939) found that the potent FSH from sheep glands is rich in carbohydrate and glucosamine. McShan and Meyer (1940) stated that their FSH preparations contain about 20% glucose.

The orcinol method was employed for the estimation of the hexose content and the Elson-Morgan procedure for hexosamine. Results showed that pure FSH contains approximately 1.3% hexose and 0.6% hexosamine. The nature of the sugar in the hormone has not yet been investigated. It may be recalled that the carbohydrate in pig FSH is mannose as identified by Gurin (1942).

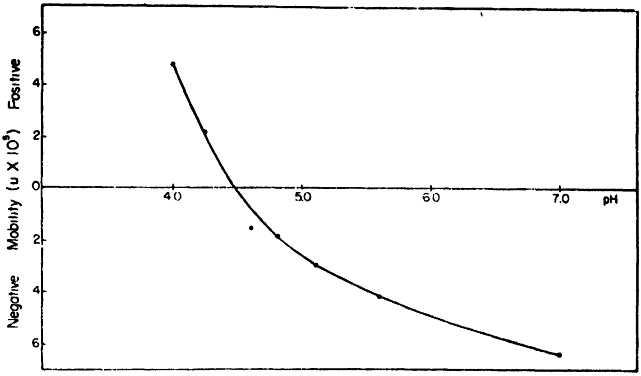

FIG. 4. Electrophoretic mobility of pituitary follicle-stimulating hormone as a function of pH at 2°C.

The tryptophane content of the hormone has been determined by the method of Horn and Jones (1943) and found to be 0.6% as an average of many determinations. The amount of tyrosine in the hormone was estimated by the procedure of Bernhart (1942) and it was shown to contain 4.4% tyrosine. Qualitative analysis indicated that the hormone has cystine and contains no cysteine.

From electrophoretic mobilities at different pHs, the isoelectric point of FSH is estimated to be approximately at pH 4.5 (Fig. 4). It is of interest to note that Chow (1943) has estimated the isoelectric point of his purified pig FSH to be about at pH 4.8.

Ultracentrifugal study of one specimen of FSH in an ultracentrifuge at 165,000 times gravity gave a sedimentation constant, $S_{20°,w}$, of 4.3 S. Without the values of diffusion constant and partial specific volume, an approximate molecular weight of FSH may be estimated to be 70,000.

Table V summarizes the known physicochemical data on FSH isolated from sheep pituitary glands.

d. Effect of Various Agents on FSH Activity. As already mentioned, FSH activity in certain anterior pituitary extracts was not destroyed by tryptic digestion (Chen and van Dyke, 1939; McShan and Meyer, 1938). Later, Chow *et al.* (1939) found that if digestion was carried to 61 to 75% with either crude or crystalline trypsin FSH activity was abolished. The same investigators also showed that crystalline chymotrypsin reduces the biological activity of FSH.

McShan and Meyer (1940) found that ptyalin and takadiastase destroy FSH activity. These results were taken to support the conclusion that a carbohydrate residue in the FSH molecule may play an important role in its biological activity.

TABLE V

Physicochemical Characteristics of Sheep Follicle-Stimulating Hormone

C, %	44.93
H, %	6.67
N, %	15.10
Tyrosine, %	4.3
Tryptophan, %	0.6
Hexose, %	1.3
Hexosamine, %	0.6
Isoelectric point, pH	4.5
Sedimentation constant, $S_{20°,w}$, S	4.3
Molecular weight	(70,000)

When a solution of crude FSH preparation was reacted with ketene at pH 5.7 for 30 minutes, it was found that the FSH activity was greatly reduced (Li *et al.*, 1939).

Fraenkel-Conrat *et al.* (1939) treated purified FSH preparations with cysteine at pH 7.7 for 2 days and found that the follicle-stimulating activity was diminished. These results were confirmed by McShan and Meyer (1940). It was concluded that -S-S- linkages are important for the hormone's biological activity.

The follicle-stimulating potency in anterior pituitary extracts was known to be comparatively stable; in solutions of pH 7 to 8 the activity is retained at 75°C. for 30 minutes (McShan and Meyer, 1940) but it is destroyed at 60°C. for 15 minutes in 50% alcoholic solution.

When 1 mg.% solution of the hormone in saline was put into a water bath of 75°C. for 30 minutes, it was found that no precipitation occurred but FSH activity was almost completely abolished. If the same solution was kept at 60°C. for 30 minutes, the follicle-stimulating activity was essentially unchanged. Thus the conclusion of McShan and Meyer cannot be confirmed by our experiments. It is probable that the presence of inert proteins may protect FSH from destruction by heat.

III. PREGNANT MARE SERUM GONADOTROPIN (PMSG)

1. *General*

In 1930, Cole and Hart, and Zondek independently discovered the presence of a gonad-stimulating hormone in the blood of pregnant mares. The hormone appears in the blood serum during the period between the thirty-seventh and forty-seventh days of pregnancy; it reaches a maximum concentration between the fiftieth and eightieth days (Fig. 5).

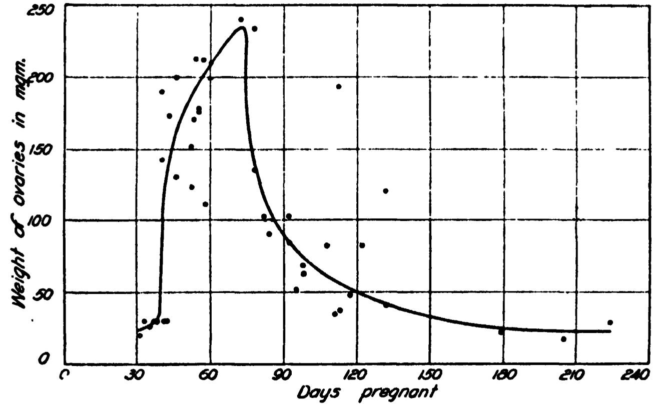

FIG. 5. The graph shows average weights of ovaries of rats injected with serum taken from mares at various stages of pregnancy. Weights include ovaries with oviducts and barsae attached. (From Cole, H. H., and Hart, G. H. *Am. J. Physiol.* **93**, 57, 1930.)

Evans *et al.* (1933b) confirmed these findings of Cole and Hart and also showed that blood serum taken from mares with fetuses of crown-rump length of from 2.5 to 18.0 cm. was usually potent in gonad-stimulating hormone. Catchpole and Lyons (1934) further investigated the relation between size of fetus and hormone concentration in the serum and found that the hormone is rarely present before the fetal crown-rump length of 2.0 cm. and rises to give frequently maximal values at 3.5–6 cm. The same investigators concluded that the hormone is secreted from chorionic epithelial cells and is not of hypophyseal origin as was believed earlier. Cole and Goss's (1943) later experiments suggested that the hormone may originate in the uterine endometrium.

The biological behavior of PMSG resembles an artificial mixture of FSH and ICSH (Levin, 1944) and for this reason it was believed that PMSG consists of two gonadotropic components. In fact, Evans *et al.* (1936) and Hellbaum (1937) claimed an actual separation of PMSG into follicle-stimulating and luteinizing fractions. These results have not been confirmed and later were refuted by Cole *et al.* (1940) who conclusively showed that the gonadotropic activity of mare serum is dependent upon a single gonadotropin.

2. *Methods of Purification*

The first attempt to purify PMGS was described in 1931 by Goss and Cole who discovered that the active principle was nondialyzable and could be fractionally precipitated by Na_2SO_4. Evans *et al.* (1933a) utilized aluminum hydroxide as an adsorption agent and this was applied by Gustus *et al.* (1936) to achieve further purification. Catchpole and Lyons (1934) employed acetone fractionation but failed to separate the hormone from the serum proteins. Later Cartland and Nelson (1937) developed a method which consists essentially of fractional precipitation with acetone or alcohol at different pH and achieved an 1800 fold purification of PMGS in terms of the original plasma. Goss and Cole (1940) reported a procedure somewhat modified from the method of Cartland and Nelson and obtained a highly purified PMSG preparation which assayed as high as 7000 I.U. (International Unit)* per milligram of protein. A more active preparation (12,500 I.U./mg.) of the hormone has been recently obtained by Rimington and Rowlands (1944). These preparations, however, have not been subjected to various tests for their chemical purity.

a. Procedure of Goss and Cole. With some modifications of the method proposed by Cartland and Nelson (1937), Goss and Cole (1940) developed a procedure to concentrate PMSG from the blood collected from mares pregnant 65 to 75 days. The following is taken directly from their paper.

The serum is first adjusted to pH 9, and an amount of acetone equal to 95% of the original volume of serum is slowly added with vigorous stirring. The precipitated proteins are allowed to stand 12 to 18 hours at room temperature and then separated out by means of flannel filter bags in a press. The pressed precipitate is washed once with a volume of 50% acetone (Sp. gr. 0.937 at 20°C.) equal to the original serum, again pressed, and discarded. The combined filtrates are carefully adjusted to 50% acetone concentration; dilute hydrochloric acid is added to bring the pH to 6.0; and the solution is then allowed to stand at 2°C. overnight. The inactive precipitate forming here is best removed with a supercentrifuge. The filtrate is brought to 70% acetone concentration and allowed to settle for 2 to 4 days at 2°C. The 70% acetone precipitate is now removed by centrifuging and is taken up in a volume of 40% neutral acetone equal in volume to one-fifteenth of the original serum. The insoluble material left behind may carry down some active material, which may, if desired, be worked up with other less potent preparations. It is separated by centrifuging, and the clear filtrate carefully adjusted to 50% acetone concentration and pH 5.5. The precipitate forming here is removed and discarded. The pH is now adjusted to 4.5, whereupon the active material separates as a slight precipitate and, after standing in the cold, is removed by centrifuging. It may be taken up in distilled water and kept frozen, or it may be preserved as a powder.

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* For the definition of International Unit of PMSG, see Thayer (1946).

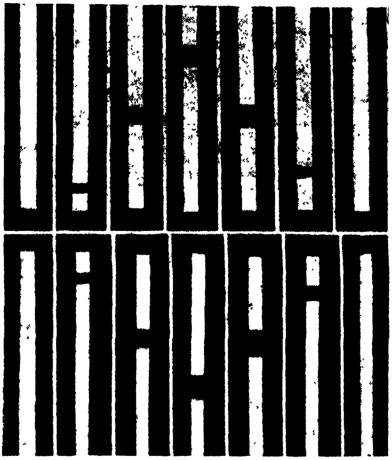

FIG. 6. Schlieren bands of pregnant mare serum gonadotropin. Exposures were made at 20-minute intervals and the current was reversed after the fourth exposure. (From Li, C. H., Evans, H. M., and Wonder, D. H. *J. Gen. Physiol.* **23**, 733, 1940.)

Li *et al.* (1940a) have examined a PMSG preparation obtained by the method just described in a Tiselius eletrophoresis apparatus and found that the preparation gave rise to a sharp boundary, indicating a high degree of purity (Fig. 6). The homogeneity of the hormone has not been examined from the standpoint of ultracentrifuge and solubility studies.

b. Procedure of Rimington and Rowlands. Rimington and Rowlands (1941) used metaphosphoric acid to remove the inert proteins from active sera and adsorbed the active material with benzoic acid. In 1944, they succeeded in preparing a material assaying 12,500 I.U./mg. by varying ethanol concentrations at different pH. The following is a summary of this procedure:

1. The serum is diluted with an equal volume of water and 0.3 *N* HPO_3 is added until the pH is about 3.6. The mixture is passed through a super-centrifuge and the precipitate is washed with 0.01 *N* HPO_3.

2. The combined filtrate is neutralized and mixed with a solution of sodium benzoate. The mixture is next adjusted to pH 4.6 with 3 *N* HCl and centrifuged.
3. Acetone is added to the precipitate; after collection, the insoluble material is washed once with 80% aqueous acetone.
4. The precipitate is dissolveed in water and NaOH is added to adjust to pH 7.0. For each 540 cc., 500 cc. ethanol and 5% acetic acid in 50% ethanol are added until the pH is 4.7–4.8. The precipitate is removed by centrifugation; to the supernatant 0.5 volume of ethanol is added to 66% and the whole is chilled overnight. The precipitate may be washed once each with absolute ethanol, acetone, and ether, then dried *in vacuo*.
5. The dried powder is next extracted with 50% ethanol containing buffer pH 4.8, removal of material insoluble in 56% ethanol at pH 6.5.
6. Finally, the supernatant is made to 66% ethanol and adjusted to pH 1.5. The precipitate formed is the purified hormone.

From 5.5 liters of pooled pregnant mare serum, the yield is approximately 20 mg.

3. Physicochemical Properties

a. Elementary Analysis and Amino Acid Data. Li *et al.* (1940a) have reported that their PMSG preparation contains 10.6% nitrogen, 0.46% amino nitrogen, 3.54% tyrosine and 1.37% tryptophan. J. S. Evans *et al.* (1942) determined the sulfur content in three preparations of PMSG with potency 800 I.U.–1800 I.U./mg. and found that it varies from 0.50 to 0.85%. The same investigators estimated the cystine content to be 1.96 to 2.78%, assuming that the hormone has no cysteine. In another publication, J. S. Evans and Hauschildt (1942) analyzed the basic amino acids in a PMSG preparation of 800 I.U./mg. and gave the following values: lysine, 8.8%, arginine, 2.1%, and histidine, 3.25%.

b. Carbohydrate Content. The sugar content in a PMSG specimen prepared by the method of Goss and Cole is 14.1% (Li *et al.*, 1940a). Gurin (1942) later identified the sugar as galactose and gave 15.6% in one preparation assaying 3300 I.U./mg. and 17.6% in another sample of 4000 I.U./mg. Rimington and Rowlands (1944) noted that their preparation contains 16.9% hexose which corresponds with the same value (14.5%) for a preparation prepared from normal horse serum by similar procedure. They concluded that carbohydrate content is no measure of gonadotropic activity of PMSG.

The hexosamine content in PMSG preparations has also been determined. The hexose: hexosamine ratio was found to be 2:1 (Gurin, 1942; Rimington and Rowlands, 1944).

c. Isoelectric Point. The electrophoretic mobility of a purified PMSG at various pH has been determined by Li *et al.* (1940a). The isoelectric point of the hormone was found to be at pH 2.60–2.65 and the value $du/dp\text{H}_0 = 4 \times 10^{-5}$. Table VI presents a summary of physicochemical properties of purified PMSG preparations.

TABLE VI

Physicochemical Characteristics of Pregnant Mare Serum Gonadotropin

N, %	10.6, 10.8
S, %	0.50, 0.85
Amino N, %	0.46
Tyrosine, %	3.54
Tryptophan, %	1.37
Arginine, %	2.1
Lysine, %	8.8
Histidine, %	3.25
Galactose, %	14.1, 15.6, 17.6, 16.9
Hexosamine, %	8.4
Isoelectric point, pH	2.60–2.65

d. Stability. J. S. Evans and Hauschildt (1942) observed that the gonadotropic activity of PMSG remained unchanged at pH 8.8 when a 1.0 mg.% solution was kept at 38°C. for 30 minutes. On the other hand, if the pH of the solution was 3.63 the activity decreased 50% within 10 hours. The activity was also somewhat decreased at pH 5.25 in 30 hours' incubation. Li *et al.* (1940a) found, however, that when 3 mg. of purified PMSG were dissolved in 10 cc. of 0.5 *M* HCl and kept at room temperature for 4 hours, the biological potency was not changed. The same authors also described a rapid destruction of the gonadotropic activity in NaOH solution under similar conditions and no inactivation was observed in solutions kept at 1.5°C. for 35 days.

Cartland and Nelson (1937) have studied heat stability of their PMSG preparation. A solution assaying 1 unit per cc. was divided into aliquots and kept for half an hour at 60, 70, 80 and 100°C. The percentage destruction at pH 6.0, 7.0, and 8.0 respectively was as follows: at 100°C., complete loss of potency in all cases; at 80°, 96, 88, and 73%; at 70°, 55, 8 and 11%; at 60°C., no destruction. These results indicate that there is a greater heat stability in neutral or slightly alkaline solution. In a recent study of Rimington and Rowlands (1944), a pH 5.9 solution of PMSG containing 210 I.U./cc. was incubated at 60° for 2 days; 50% of the potency was lost. As shown in Table VII, the rate of inactivation is approximately the same in the presence of air, oxygen, or nitrogen. Apparently, the destruction is not an oxidative process.

Rimington and Rowlands (1944) studied further the effect of pH upon stability at 37°C. It was found that inactivation occurs spontaneously and more rapidly in the presence of acid or alkaline buffers. For instance, approximately half of the original gonadotropic potency is lost in 2 days at pH 4.5 acetate buffer, whereas the water solution (0.1 mg. hormone/cc.) remains unchanged.

TABLE VII

Rate of Inactivation of PMSG at 60° and pH 5.9 in Oxygen, Nitrogen and Air Respectively

Time	Potency of solution in Oxygen	 Nitrogen	 Air
hrs.	I.U./cc.	I.U./cc.	I.U./cc.
0	210	210	210
24	113	160	170
48	105	127	80
72	45	60	43
96	31	28	40

(From Rimington, C., and Rowlands, I. W. *Biochem. J.* **38**, 59, 1944.)

It is generally known that protein substances are most stable in their dry state. But this is not the case with PMSG. Li *et al.* (1940a) have shown that the gonadotropic potency of a dry sample of PMSG assaying 1000 I.U./mg. decreased to one-half after a storage of 2 weeks and it became stable at this potency. Rimington and Rowlands (1944) have also reported similar loss of activity on storage. There are no apparent reasons for the loss of activity in the dry state.

4. Effect of Various Agents on PMSG Activity

a. Reactions with Ketene, Nitrous Acid, etc. The gonadotropic activity of PMSG is destroyed by ketene (Li *et al.*, 1939), nitrous acid (Bischoff, 1942) and formaldehyde (Cartland and Nelson, 1937; Bischoff, 1941). Although these studies did not correlate the loss of free amino groups with physiological potency, it may be inferred that free amino groups play an important role in the gonadotropic function of PMSG.

b. Reactions with Cysteine and Other Reducing Agents. Complete inactivation of PMSG occurs upon prolonged treatment with a large excess of cysteine (Fraenkel-Contrat *et al.*, 1940; J. S. Evans *et al.* 1942). When the hormone reacts with sodium cyanide at pH 8.0 for 30 minutes, some inactivation was observed (J. S. Evans *et al.*, 1942). The effect of other reducing agents on the activity of PMSG has also been studied by J. S. Evans *et al.* (1942). These data seem to indicate that reduction of the disulfide linkages in the hormone molecule results in a loss of PMSG activity.

c. Effect of Enzymes. Trypsin destroys the gonadotropic activity of PMSG (Goss and Cole, 1931; Evans *et al.*, 1933; Cartland and Nelson, 1937; and J. S. Evans and Hauschildt, 1942). The hormone is also inactivated by pepsin, chymotrypsin, papain and carboxypeptidase (J. S. Evans and Hauschildt, 1942). The fact that the biological activity of the hormone is rapidly abolished by carbohydrate-splitting enzymes (ptyalin and takadiastase) suggests that the intact carbohydrate residue of the molecule is essential for a gonadotropic action.

d. Effect of Urea. Urea is recognized as one of the denaturing agents for proteins. It should be of interest to investigate whether a denatured biologically active protein retains its physiological function. Bischoff recently (1944, 1946) studied the action of urea on the gonadotropic activity of PMSG in some detail. It was found that when a dilute solution of PMSG in 40% urea was kept at 37.5° for 22.5 hours, 63% of the biological potency was lost. On raising the temperature to 80°C., similar loss of activity occurred in 35 minutes. At an intermediate temperature (67°C.) a loss of only 37% of the activity was observed in 360 minutes. Bischoff further showed that the inactivation followed a first order kinetics. From reaction rates at different temperature, the same author estimated the energetics of the denaturation reaction for PMSG and found that the change of free energy, ΔF, at 37° is 25, 200 calories per mole and the entropy, ΔS, +61 calories per degree per mole. These values represent the characteristics of a classic protein denaturation.

IV. Human Chorionic Gonadotropin (HCG)

1. *General*

The occurrence of a gonadotropic principle in human pregnancy urine was first described by Aschheim and Zondek in 1927. Browne and Venning in 1936 found that the urinary excretion of HCG reaches its maximum concentration during the period between the 60th and 80th days of pregnancy. This is also concluded by Evans *et al.* (1937), Smith and Smith (1937), and others. Many methods have since been proposed to concentrate HCG from these urines. Zondek and Aschheim (1928) precipitated the active principle with alcohol. Other investigators employed adsorbents for direct adsorption of the hormone from the urine. Benzoic acid has proved to be the most effective and useful reagent (Katzman and Doisy, 1932); using the benzoic acid precipitate as the starting material, Gurin *et al.* (1939) have succeeded in preparing a highly purified product which behaves as a single substance in electrophoresis, ultracentrifuge and diffusion. Katzman *et al.* (1943) obtained a preparation with the same biological potency as the material of Gurin *et al.* by chromatographic adsorption on Permutit followed by elution with 10% ammonium acetate in aqueous alcohol; no physical test for purity was carried out, though it may have the same purity as Gurin's preparation. Recently Claesson *et al.* (1948) described a procedure for the crystallization of an electrophoretic homogeneous HCG preparation assaying 6000–8000 I.U./mg.

The biological properties of HCG are distinct from other gonadotropins (Levin, 1944). In hypophysectomized rats it does not cause follicular growth and maturation and produces only hypertrophy of interstitial cells. Thus, the increment of ovarian weight in hypophysectomized animals by HCG stimulation is very slight. Even when tested in normal immature rats, HCG produces only a limited increase in the ovarian weight. The effect of HCG in hypophysectomized male rats is the maintenance or repair of the testicular interstitial cells. If the treatment is begun immediately after hypophysectomy, the tubular structures are also maintained and spermatogenesis continues in an approximately normal manner. Thus, the physiological function of HCG is similar to that of the interstitial cell-stimulating hormone of the anterior pituitary. It will be noted that chemically these two hormones are very different.

2. Methods of Purification

a. Procedure of Gurin, Bachman, and Wilson. In 1939, Gurin *et al.* described a method for preparing a highly purified sample of HCG from pregnancy urines which were collected between the 50th and 90th days following the last menstrual period. Their procedure, carried out at 0.5°C., may be summarized in the following steps:

> 1. The pooled urines are treated with benzoic acid according to the method of Katzman and Doisy (1932). The benzoic acid in the precipitate is removed with acetone. The insoluble residue is dried and yields approximately 500 mg. from each liter of urine.
> 2. The residue is next extracted with 50% ethanol at pH 6.0. The extract is treated with 2 volumes of cold absolute ethanol to precipitate the hormone.
> 3. The material precipitated by alcohol is extracted three or four times by stirring 30 minutes with $1x$ to $2x$ cc. of 0.3 M sodium acetate-acetic acid buffer in 50% ethanol at pH 4.8. After centrifugation, the combined extracts are precipitated with an equal volume of cold absolute alcohol and chilled for a few hours. The precipitate is dried and yields approximately 8 mg. from each liter of urine.
> 4. One hundred milligrams of the product just described is dissolved in 2 cc. of ice-cold water and adjusted to pH approximately 5.

The solution is next shaken vigorously for 2 hours with 0.5 cc. chloroform. The chloroform emulsion is washed several times with 0.5 cc. water. The combined clear aqueous solutions are dialyzed and dried.

The product assays 6000 I.U./mg. From 1 liter of urine approximately 6 mg. of this highly active material may be obtained. It was found that the hormone behaves as a single protein in electrophoresis and ultracentrifuge (Gurin *et al.*, 1940a, b; Lundgren *et al.*, 1942). The solubility test for its purity has not been carried out.

b. Procedure of Katzman et al. The use of permutit to adsorb HCG from the urine was first introduced by Lejwa in 1932. Later, Katzman and his colloborators (1943) employed the same adsorbent and obtained a highly purified preparation of HCG assaying 8500 I.U./mg. Their procedure is briefly as follows:

Urine obtained during the first half of pregnancy is chilled, filtered, and acidified to pH 3.5 with glacial acetic acid. After filtration, the clear filtrate is percolated through a column containing Permutit. Adsorption is complete when 10 liters of urine per hour are passed through a column having a diameter of 4 inches and containing 2 kg. of Permutit. The column is then washed with cold distilled water until the washings are neutral and practically colorless. The hormone is then eluted with 38% ethanol containing 10% ammonium acetate. The hormone in the eluate is fractionally precipitated with ethanol. The bulk of the activity precipitates at 70 or 75% ethanol concentration. The hormone thus obtained contains a potency as high as 8500 I.U./mg.

This method of Katzman *et al.* appears very simple and should be practical in obtaining a large amount of the hormone for further biological and chemical investigations.

c. Procedure of Claesson et al. A method for the preparation of crystalline, electrophoretically homogeneous HCG was recently described by Claesson *et al.* (1948). Their procedure may be briefly described in the following steps which are performed in a cold room at 4°C.:

1. The active principle in the urine of pregnant women (1–3 months of pregnancy) is adsorbed with benzoic acid similar to the procedure of Katzman and Doisy (1932). After extraction with acetone the residue is taken up in a *M*/15 acetate buffer of pH 4.8.
2. To this pH 4.8 extract ethanol is then added to a concentration of 85%. After centrifugation, the precipitate is reextracted with buffer and again precipitated with 85% ethanol. This step yields 25–30 mg. purified HCG from 1 liter of urine assaying 4000–6000 I.U./mg.

3. Five hundred milligrams of this material is dissolved in 50 cc. *M*/75 phosphate buffer of pH 7.4. After the removal of insoluble residue, a 10% aqueous protamine solution is added drop by drop until no more precipitate appears. The protamine precipitate does not possess any biological activity and is discarded.
4. The clear supernatant is then adjusted to pH 3.5 and brought to 50% ethanol by the addition of an alcohol solution containing 5% *m*-cresol. After 2 hours of standing the ethanol concentration is increased to 60%. The hormone first precipitates out as an amorphous substance and begins to crystallize in long thin rods or needles after standing for 24 hours.

The authors claimed that "by means of electrophoretic studies, using phosphate buffer of pH 7.0 and ionic strength of 0.1, in contrast to the several different fractions previously obtained only one component could be demonstrated, even after 2 hours." They did not present the schlieren scanning patterns obtained from the electrophoretic experiments. Since the crystalline preparation has not been subjected to ultracentrifuge and solubility tests and since crystallinity does not mean absolute purity, the HCG preparation of Claesson *et al.* may yet contain a small amount of contaminating substance(s).

3. Physicochemical Properties

a. Molecular Kinetic Data. Lundgren *et al.* (1942) have studied the ultracentrifugal and diffusion behavior of Gurin's HCG preparation. The data indicated that the hormone is essentially homogeneous; the sedimentation constant, S_{20}, is 4.3 S and the diffusion constant, D_{20}, 4.4×10^{-7} cm.^{2}/sec. With the value of partial specific volume, 0.76, the molecular weight is computed to be 100,000.

b. Electrophoretic Data. One electrophoretic experiment in a Tiselius apparatus was carried out with the HCG preparation of Gurin *et al.* in a pH 7.0 phosphate buffer of ionic strength 0.10 (Gurin *et al.*, 1940a, b). It was found that one moving boundary appeared with a mobility of 4.85×10^{-5}. In order to establish the fact that a protein preparation is electrophoretically homogeneous, experiments should be conducted in several buffers of different pH.

The isoelectric point of the hormone has been estimated in a microelectrophoresis apparatus and found to be between 3.2 and 3.3. Earlier a similar value was reported by Spielman and Meyer (1938) using a less purified preparation.

c. Chemical Composition. The nitrogen content of HCG is reported to be 12.03% (Gurin *et al.*, 1940a). Tyrosine, histidine, arginine, and tryptophan were present in the hormone,* but no quantitative data were given; cystine has not been detected. Since the hormone has 1.96% sulfur, it must contain a high percentage of methionine.

TABLE VIII

Physicochemical Characteristics of Human Chorionic Gonadotropin

N, %	12.03
S, %	1.96
Galactose, %	10.7
Hexosamine, %	5.2
Sedimentation constant, S_{20}, S	4.3
Diffusion constant, D_{20}, cm.^{2}/sec.	4.4×10^{-7}
Partial specific volume	0.76
Molecular weight	100,000
Isoelectric point, pH	3.2–3.3

Like other gonadotropins, HCG contains carbohydrate in the hormone molecule. Gurin *et al.* (1940a) identified the sugar as galactose by both analytical and isolation procedures. The presence of hexosamine has also been demonstrated, but it has not been identified. The amount of galactose and hexosamine in Gurin's HCG preparation is 10.7% and 5.2% respectively. It may be noted that the galactose/hexosamine ratio is 2:1, which is about the same as for PMSG.

Table VIII summarizes the physicochemical data on human chorionic gonadotropin.

d. Solubility and Stability. Highly purified HCG preparations are highly soluble in water but insoluble in usual organic solvents. The hormone is precipitated by 70% alcohol or acetone from neutral or slightly acid solutions. The usual protein precipitants (picric, picrolonic, flavianic, trichloroacetic or sulfosalicylic acids) do not cause HCG to precipitate, but phosphotungstic acid and phosphomolybdic acids do.

A dilute solution of HCG loses gonadotropic activity rapidly on storage at 0°C. Concentrated solutions appear to be more stable. Katzman *et al.* (1943) stated that $MgCl_2$, gelatin, and serum protein may protect the hormone from inactivation in solutions.

In aqueous solution, the activity of HCG is abolished by heating and by excess acid or base. The hormone is stable in glycerol solution when heated at 100°C. for an hour. The inactivation of HCG in 1% aqueous NaCl solution, pH 6.3, at various temperatures has been studied by

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* The reviewer has prepared a highly purified preparation of HCG from a crude commercial material according to the procedure of Gurin *et al.* Tyrosine and tryptophan analysis of the purified product was carried out by the method of Lugg (*Biochem. J.* **32**, 775, 1937) and it was found that the hormone contains 1.2% tyrosine and 0.15% tryptophan.

Bischoff (1946). It may be seen in Table IX that the hormone is comparatively stable at 50°C. but rapidly decreases in activity at 65°C. In contrast to PMSG, HCG is stable in the dry state.

TABLE IX

Inactivation of HCG in 1% Aqueous NaCl Solution, pH 6.3, by Heat

The hormone concentration was 100 I.U./cc.

Temperature of reaction	Time of reaction	Estimated recovery of the hormone
°C.	min.	%
65.0	5	50
	15	24
62.5	5	65
	15	35
60.7	15	66
56.9	60	56
	270	61
50.0	1550	28

(From Bischoff, F. *J. Biol. Chem.* **165**, 406, 1946.)

4. *Effect of Various Agents on HCG Activity*

It has been reported that trypsin, chymotrypsin, papain, and salivary enzyme preparations destroy the gonadotropic activity of HCG (Abramowitz and Hisaw, 1939). It was earlier claimed that the hormone is inactivated by trypsin but not by pepsin (Reiss and Haurowitz, 1929; Wiesner and Marshall, 1931). This fact, that the activity of HCG is resistant to peptic digestion, has not been carefully studied with the purest HCG preparation. It is probable that certain peptide fragments after pepsin hydrolysis may possess some gonadotropic potency.

Meyer (1937) allowed the hormone to react with iodine in slightly acid solution and obtained a highly active preparation which was shown to be an iodinated compound. It was found that if the iodine reaction were conducted in neutral solution, its hormonal activity was abolished.* From our knowledge of protein-iodine reactions, it is probable that the tyrosine residues in HCG react readily with iodine in neutral solution, thus causing the loss of biological activity. On the other hand, since the tyrosine groups cannot be iodinated in acid solution, the iodo-HCG of Meyer must be due to the reaction of iodine with other residues in the hormone molecule.

As has already been mentioned, HCG contains no cystine. It is therefore not surprising to note that cysteine does not inactivate the hormone (Bischoff, 1940). However, if a large excess of cysteine was allowed to react with HCG for a longer time, some inactivation occurred. This may be caused by the process of denaturation rather than by actual reaction with the reducing agent. It will be seen that denaturation of the protein molecule decreases the gonadotropic activity of HCG.

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* Unpublished results of the reviewer.

Ketene inactivates the hormone slowly (Li *et al.*, 1939). The authors compared their reaction with other studies with ketene and concluded that the decrease in potency is probably due to the acetylation of the tyrosine phenolic groups. The hormone is stable in 0.1 *N* nitrous acid at 2°C. and is relatively stable in 0.5 *N* acid (Bischoff, 1942). These data may be taken to support the conclusion that free amino groups are not essential for the gonadotropic action of HCG.

The denaturation of HCG by exposure to 40% urea solution of pH 7.2 at 37.5°C., has been studied by Bischoff (1944, 1945a, 1945b, 1946). Under these conditions, 80% of the hormonal activity was lost in 60 minutes and the rate of inactivation was a first order reaction. From the inactivation rates at various temperatures, Bischoff has calculated the following constants: heat of activation for the denaturation, 27,100 calories per mole, and the corresponding entropy of activation, 14.1 calories per degree per mole.

When a dilute solution of HCG was exposed to another denaturing agent, sodium lauryl sulfate, there was no loss of gonadotropic activity (Bischoff, 1945c). On the other hand, HCG was shown to be inactivated by x-rays (Hochman *et al.*, 1947).

V. Summary

The four gonadotropic hormones (ICSH, FSH, PMSG, and HCG) discussed in this paper have been prepared in different degrees of purity; one of them (HCG) has been prepared in crystalline form. Perhaps the purest of all is the ICSH isolated from sheep and pig glands; its homogeneity has been established by the solubility test which is the most reliable criterion of purity for a protein. Although highly active pregnant mare serum gonadotropic preparations have been obtained, physicochemical tests for their purity have not been carried out. Therefore this cannot be admitted into the group of pure hormones.

These gonadotropic hormones are glycoproteins; in addition to the amino acid constituents, these substances contain hexose and hexosamine. Destruction of the carbohydrate residue by enzymes causes inactivation of the hormones. Similarly, modification of protein groups decreases the gonadotropic activity. Both the carbohydrate and protein parts are essential for the biological action. There are no indications of the occurrence of prosthetic groups in these hormones. The complete compositions of the gonadotropins have not been investigated.

The availability of pituitary glands has limited the practical applications of FSH and ICSH in human therapy. In addition, there is evidence for the formation of antihormone during the administration of these gonadotropins. This may be avoided by the use of hormone preparations isolated from human tissues or fluids. In the case of ICSH, we have chorionic gonadotropin which acts biologically as an interstitial-cell stimulator, but we do not have purified fractions having FSH activity. Further studies to obtain highly purified gonadotropin from blood or urine of ovariectomized and postmenopausal women and of normal men are therefore desirable.

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The Chemistry and Physiology of Adenohypophyseal Luteotropin (Prolactin)

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I. INTRODUCTION

The historical development of knowledge regarding the chemistry and physiology of adenohypophyseal (anterior pituitary) luteotropin began with studies of the role of the hypophysis in the regulation of milk secretion. The hypophysis was early implicated in the process of lactation by the observations that hypophysectomy in the pregnant female prior to parturition will almost uniformly prevent normal lactation, although normal young are born. Also, hypophysectomy during lactation will result generally in a cessation of milk secretion. Moreover, various experimental procedures which normally lead to lactation are incapable of inducing milk secretion in animals from which the hypophysis has been removed. All of these data suggest a role for adenohypophyseal secretion in the initiation and maintenance of lactation. The literature bearing on this topic has been reviewed in detail (Nelson, 1936; Folley, 1938, 1940; Turner, 1939).

The first definite evidence of the capacity of hypophyseal extracts to influence lactation was provided by Stricker and Grueter in 1928. These authors were able to induce lactation in ovariectomized-pseudopregnant rabbits by the injection of crude adenophypophyseal extracts. It was also observed by these authors that a single injection of their extract was capable of re-initiating lactation in rabbits in which lactation had ceased 10 to 15 days earlier. These observations have been confirmed and extended by numerous investigators, and the data have also been reproduced with highly purified fractions of adenohypophyseal extracts (Nelson, 1936; Riddle and Bates, 1939; Turner, 1939; Folley, 1940).

The active principle of adenohypophyseal secretion and extracts which is responsible for the initiation and maintenance of lactation, and for other physiological responses which will be described below, has been given a variety of names, namely, lactogenic hormone, prolactin, galactin, mammotropin, lactogen, and luteotropin. Although the terms lactogenic hormone and prolactin have been employed commonly in the literature, these names focus attention on only one of the physiological roles of this adenohypophyseal hormone, namely, its participation in the secretion of milk. In view of the broader physiological implications for this hormone, which will be considered in this review, it would appear desirable to adopt the more general name, luteotropin, to designate the hypophyseal principle referred to formerly chiefly as prolactin or the lactogenic hormone. While it is true that the term, luteotropin, also is not completely satisfactory because it likewise is suggestive of a particular type of physiological function, it does seem to be less restricted than prolactin or lactogenic hormone. The term, luteotropin, is to be distinguished from the designation used for the hormone of the adenohypophysis which stimulates formation of luteal tissue and corpora lutea, namely the substance known as either luteinizing hormone, interstitial cell stimulating hormone, or metakentrin.

II. Methods of Assay

The existence of a biocatalytically active substance in living cells is first recognized generally as a result of the description of the physiological processes whose rates are affected by the catalyst. As a result of such experimental observations, a method of assessing the concentration of the material is evolved and serves as a fundamental requisite for studies designed to fractionate and eventually isolate in pure form the active principle. The literature dealing with the chemistry and properties of the two large groups of biocatalysts, namely, the enzymes and the hormones, is replete with examples of the basic need for the development of precise methods of assay as a preliminary to further chemical and physiological studies.

1. Crop Gland Methods

The most widely used methods of assay of the potency of preparations of luteotropin, and of fluids for their content of this hormone, are based upon the observation made in Riddle's laboratory (Riddle and Bates, 1939) that when solutions containing luteotropin are administered intramuscularly to young pigeons, a proliferative hypertrophy of the crop-sacs occurs. The assay based on this fact has been termed the systemic or crop-sac weight method, inasmuch as the increase in weight of the crop-gland, due to the proliferative action of luteotropin, is generally determined. McShan and Turner (1936) have adapted this physiological response to a systemic minimal stimulation method, in which the degree of crop-sac hypertrophy is estimated on the basis of inspection of the excised gland. This hypertrophy may be limited experimentally to only a small area of crop-sac in the pigeon by injecting small doses of luteotropin-containing extracts intradermally directly into the skin overlying the crop-gland (Lyons and Page, 1934–35). The latter procedure affords a method of detecting very small quantities of the hormone, and has been termed the local or microassay method.

a. Systemic or Crop-Sac Weight Method of Assay. This method was developed and has been studied extensively in Riddle's laboratory by Bates. The review by these authors (Riddle and Bates, 1939) includes a detailed description and critical analysis of the technique, as well as some consideration of the systemic minimal stimulation method of McShan and Turner (1936). Folley *et al.* (1940) and Hall (1944a, b) have also subjected the crop-sac weight method to critical examination.

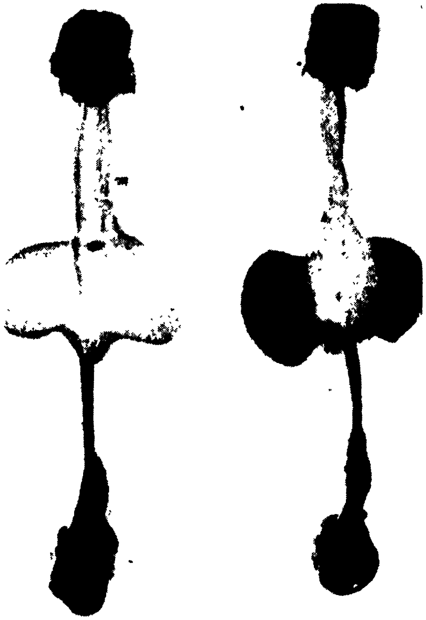

FIG. 1. Stimulation of pigeon crop-sac by administration of luteotropin. N501, control crop-sacs of normal adult ring dove. N516, crop-sacs of a normal ring dove after injection for 10 days with luteotropin. (After Riddle, O., and Bates, R. W., in Sex and Internal Secretions. 1939. Edited by E. Allen, 2nd ed., Williams & Wilkins Co., Baltimore, pp. 1088–1117.)

The bird most commonly used has been the White Carneau pigeon in the age range of 2 to 3 months. Bates *et al.* (1939a) showed the importance of the racial factor in the pigeon crop-sac method of assay by demonstrating that in terms of luteotropic units, an extreme variation of fivefold was found among six races of pigeons and of eightfold among fourteen strains of doves. These variations were still more extreme when high doses of hormone were used. Generally, the solution to be tested is injected intramuscularly once daily for 4 days with autopsy 24 hours after the last injection. The crop-glands are dissected out, scraped free of contents and adhering material, and weighed. The thickened mucosa which results from hormone administration is illustrated in Fig. 1. Under carefully controlled conditions, there is a linear relation between the crop weight and the logarithm of the dose over a considerable range of dosage. This relationship is depicted in Fig. 2, which is taken from the paper of Riddle *et al.* (1933). More recently, Hall (1944a) has presented the data plotted in Fig. 3, which suggest that the slope of the regression line (relating dose with response) in the systemic crop-weight method of assay is valid only when stimulation produced crop-sacs weighing more than 2200 to 2500 mg.

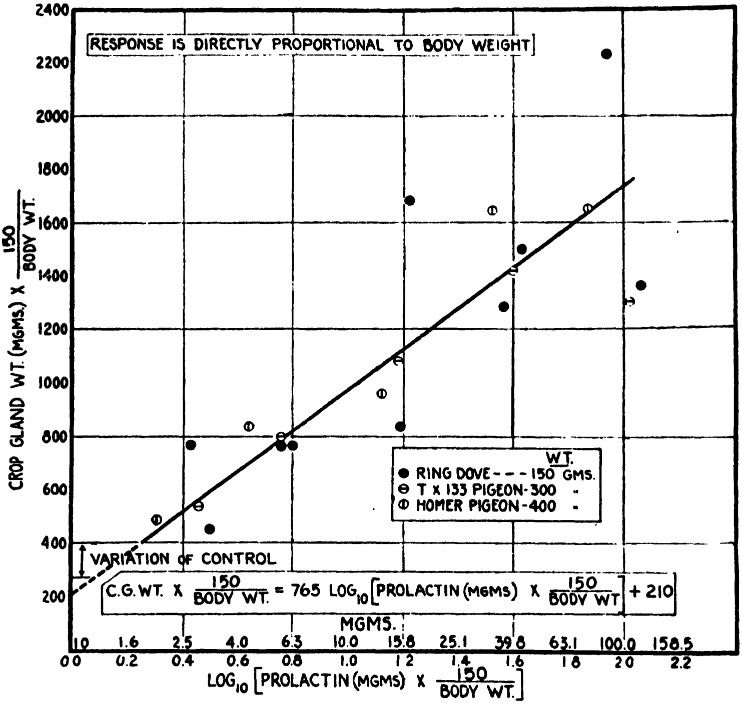

FIG. 2. Individual responses of immature doves and pigeons to a luteotropin preparation (No. 65). Points on curve represent individual birds. (After Riddle, O., Bates, R. W., and Dykshorn, S. W. 1933. *Am. J. Physiol.* **105**, 213.)

In assaying crude preparations of luteotropin or tissue extracts for their content of this hormone, it is important to note the observations of Friedman and Hall (1941) and of Hall (1944a) that muscle extracts added to purified luteotropin solutions may augment or depress the crop-sac response, depending on the proportion of muscle extract used.

The so-called minimum stimulation method of assay described by McShan and Turner (1936) uses a routine of injection which is essentially that described above, except that the crop-gland is not weighed. The dissected, cleaned crop-sac is distended and examined by transmitted light. Positive stimulation is indicated by the presence of typical parallel strands of thickened mucosa. The authors define their pigeon unit as the total amount of hormone which, when injected during a 4-day period, will cause a minimum but definite proliferation of the crop-glands of 50 ± 11% of twenty common pigeons weighing 300 ± 40 g. Figure 4 illustrates the type of response by this assay procedure. The characteristic sigmoid curve indicates extreme sensitivity to hormone in the region of 50% response. Lyons (1937) has employed a similar minimum stimulation systemic unit which he defined as the smallest amount of hormone which, when injected subcutaneously for 4 days in Silver King squabs

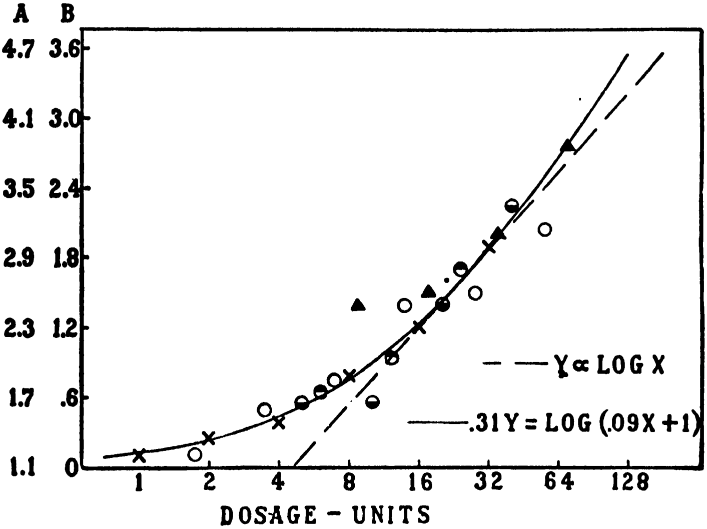

FIG. 3. Pigeon crop-sac response to luteotropin. Column A on the vertical axis represents actual crop-sac weight in grams; column B, the increase in crop-sac weight in grams over control. Column B is used in the equation $.31y = \log(.09x + 1)$, in which x is expressed in luteotropin units. The solid line is the calculated curve from the equation. The broken line is the dose relationship of Riddle and Bates (1939) who proposed that y was proportional to $\log x$. The points represent the assay of five different preparations. (After Hall, S. R. 1944. *Endocrinology* **34,** 1.)

(one month from hatching) causes, by the end of the fifth day, a positive response in the majority of five birds.

In the experience of the writer, the procedure described by Lyons has been very useful in following the purification of the luteotropic hormone, and in assessing the potency of certain purified preparations. However, establishment of precise biological potency, within the limits of the accuracy of the methods and the information available for the bioassay of luteotropin, can be accomplished most precisely by the crop-sac weight procedure. With the latter, it is important to consider the diverse variables which affect the assay; these variables have been discussed by Riddle and Bates (1939), by Bates *et al* (1939a, b) and by Bates and Riddle (1941).

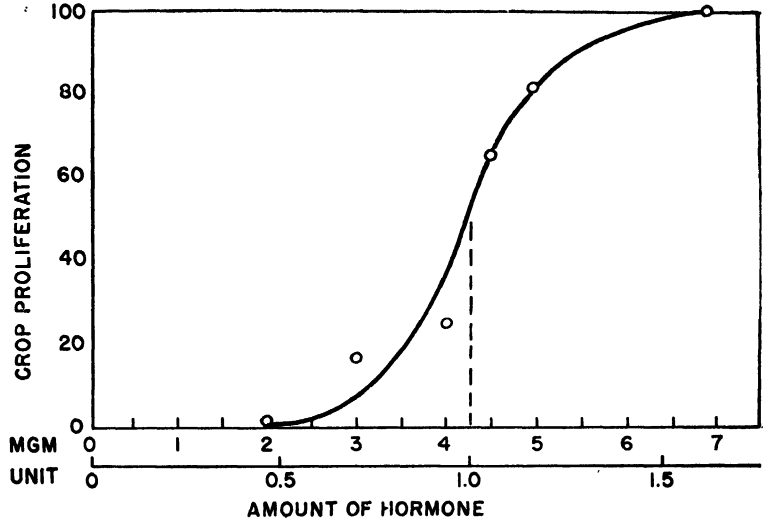

FIG. 4. Relation between quantity of hormone and percentage of positive responses in pigeons injected intramuscularly for 4 days. (After McShan, W. H., and Turner, C. W. 1936. *Proc. Soc. Exptl. Biol. Med.* **34,** 50.)

b. Local Stimulation Method. This procedure is based on the observation by Lyons and Page (1934–35) that the hypertrophy of the crop-sac which results from the systemic injection of luteotropin may be limited experimentally to only a small area of crop-sac in the pigeon by injecting small doses intradermally directly into the skin overlying the gland. The portion of the crop-sac stimulated by the intracutaneous technique is that directly under the site of injection; thus, two different dose levels or preparations can be tested in the same bird on the two opposite sides of the crop-gland. An intradermal injection is made on either side, each injection representing a total volume of 0.1 ml. The injections are made over as much as possible of the ventral and ventrolateral areas of the crop-sacs, at a time when the crops are distended with food. The feather ridges from which the feathers have been plucked afford the best injection sites. The birds are sacrificed 24 hours after the last injection, the muscle layer stripped from the crop-sacs, and the latter inspected by holding the stretched membrane to the light, or sectioned for microscopic examination. Figure 5, A–E, shows the technique used in the local injection method, and the type of response obtained. This method has

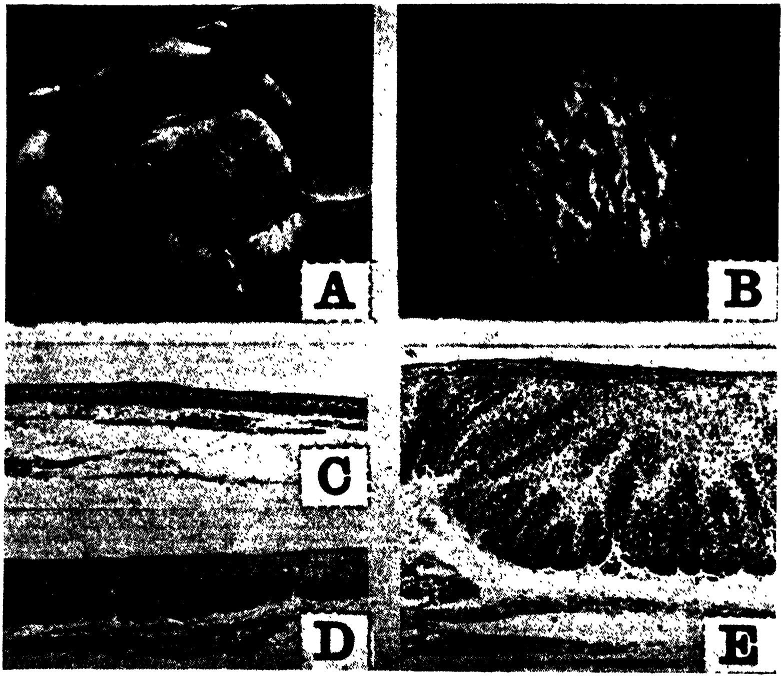

FIG. 5, A. The plucked crop-sac area of a 1-month-old Silver King squab; 0.1 ml. of solution is injected into a feather ridge at the point indicated. A different solution is tested on the opposite side.

B. The appearance of a positive "local" reaction to luteotropin when the stretched crop-sac is held to the light. ×4.

C. Section of normal crop sac membrane of a 1-month-old squab. ×80.

D. Section showing a low grade reaction obtained 48 hours after a single "local" intradermal injection of 0.1 γ of hormone. ×80.

E. Section showing high grade "local" reaction to 6.4 γ of same. ×80. (After Lyons, W. R. 1937. *Proc. Soc. Exptl. Biol. Med.* **35**, 645.)

a high degree of sensitivity, approximately one thousand times that of the intramuscular assay (Lyons, 1936–37, 1937), and is therefore useful for detecting small quantities of luteotropin. Because of this, Lyons (1937) has found this technique useful for estimating the luteotropin content of unconcentrated urine.

The local crop-sac method affords certain definite advantages in assaying materials for their luteotropin content. It has a high degree of sensitivity, and therefore can detect very small quantities of active substance. The time required for assay is shorter than in the crop-weight methods, and the response is not dependent on the size of the bird used; these are important factors in the crop-weight methods. However, the response by the local procedure is still dependent on the particular breed of bird employed. Moreover, the method has been subject to a degree of warranted criticisms which serve to emphasize additional variables that must be recognized when this technique is used. The volume of solution which is injected intradermally must be constant throughout an assay of a single preparation, or when comparing several products for their luteotropic hormone content (Bates and Riddle, 1940). This observation was confirmed by Hall (1944a), who also emphasized the importance of the exact site of the intradermal injection with respect to the underlying crop-sac. Of considerable importance also was the observation that the assay of suspensions of acetone-dried or fresh tissues, instead of clear extracts, might give data which were 100% in error. Finally, attention should be directed to the further examination by Lahr *et al.* (1943) of factors influencing the specificity of the micro method of luteotropin assay by the local crop-sac response. Pronounced wounding of the underlying epithelium of the crop, produced either by plucking of feathers incident to making local crop-sac tests for luteotropin, or by intradermal injection of nonluteotropin-containing irritants, caused a prolonged increase of mitotic rate in this epithelium with a macroscopic appearance which would be considered normally as a positive hormonal response.

2. Mammary Gland or Lactation Methods

The mammary gland or lactation methods are based on the fact that the luteotropic hormone, when administered to animals with prepared or hypertrophied mammary glands, will stimulate the secretion of milk. The chief drawback of these methods resides in the subjective evaluation of the degree of lactation, which in turn will be dependent not only upon the dose of hormone, but also on the amount of mammary tissue, its maintenance, and its blood supply. Because of the necessity of selecting animals with uniform mammary development, or the production of this by proper use of hormones, the lactation methods appear to be less desirable for routine use than the crop-sac methods.

Although the luteotropic hormone will induce lactation in numerous species (Turner, 1939), only the pseudopregnant rabbit and the hysterectomized guinea pig have been studied adequately from the standpoint of bioassay.

a. Pseudopregnant Rabbit. The use of the pseudopregnant rabbit for the assay of luteotropin was proposed by Gardner and Turner (1933). Pseudopregnancy is induced either by a vasectomized buck or by administration of gonadotropic hormone, and the mammary glands checked for proper development. The preparation to be assayed is then injected, and the condition of the lactating mammary graded qualitatively in terms of relative degree of milk production.

b. Hysterectomized Guinea Pig. Nelson (1934) described conditions for assay of the luteotropic hormone in pregnant guinea pigs which were hysterectomized between the fortieth and fifty-fifth days of pregnancy. Four to five days after operation, the animals were injected twice daily for 7 days with the preparation being assayed. The result was graded as positive if milk could be expressed from the nipples during the test period. Generally, in the case of a positive reaction, this occurred in less than 48 hours. The importance of the condition of the mammary gland in influencing this response is seen from the observation that more than two and one-half times as much luteotropin was required to induce lactation in the normal adult female guinea pig as in the hysterectomized pregnant guinea pig. Furthermore, it was found that the latter preparation responded with a markedly positive lactation response when given approximately one-fifth of the amount of hormone required to induce a comparable intensity of lactation in the pseudopregnant rabbit.

III. DISTRIBUTION IN NATURE

1. *Occurrence in Animal Tissues*

The primary site of occurrence of the luteotropic hormone is, of course, the adenohypophysis. The concentration of the hormone in this tissue will vary with periods in the life and reproductive cycle of the particular species being studied. Small concentrations of luteotropin have been reported in other tissues. The validity of these observations is, however, open to question in some instances inasmuch as the specificity of the assay method employed, as well as the effect of the presence of other tissue components, may not have been evaluated adequately. Luteotropin has been detected in neurohypophyseal tissue (Lyons, 1937;

Riddle and Bates, 1939) and, in the experience of the writer (unpublished experiments), the yield of luteotropin is much larger when whole hypophyseal glands (beef) are extracted than when an approximately equivalent weight of dissected adenohypophyseal tissue is used as starting material.

As mentioned previously, Lyons (1937) reported the detection of luteotropin in unconcentrated human urine, using the local crop test. With a similar test procedure, small amounts of the hormone have been found in postpartum human urine (Lyons and Page, 1935; Tesauro, 1936; Riddle and Bates, 1939), and in purified pregnancy urine extracts (Riddle and Bates, 1939). Lyons and Page reported that the amount of hormone excreted daily was equal approximately to that present in a single beef hypophysis. Langecker and Schenk (1936) confirmed the presence of luteotropin in urine on the basis of recovering a quantity sufficient to affect milk secretion in rabbits. The hormone has also been detected in blood. Tesauro (1936) obtained positive tests with human serum, while Leblond (1937), using the local crop-sac test, reported traces of luteotropin in the serum of lactating and pregnant mares.

Luteotropin has been reported to be present in a wide variety of other tissues: fish liver and brain (Leblond and Noble, 1937), pigeon liver (Riddle and Bates, 1939); cattle and hog liver (Rabald and Voss, 1939), young, but not mature, placental tissue (Ehrhardt, 1936), cow mammaries and milk, shortly following parturition (Geschickter and Lewis, 1936), and cystic human mammaries (Geschickter and Lewis, 1936).

2. *Species Variations*

As is true with other hormones, the content of luteotropin in adenohypophyseal tissue may show not only marked species variation, but also may differ among members of the same species, depending on the physiological state of the particular animal from which the hypophyseal tissue is obtained. Bates and Riddle (1935) found that beef and sheep glands yielded, per gram of fresh tissue, approximately equal quantities of luteotropin activity, whereas pork glands contained about one-fifteenth as much hormone. The luteotropic hormone has been demonstrated in hypophyseal tissue of the following species: mice, rats, guinea pigs, rabbits and cats (Reece and Turner, 1937a). Luteotropin has also been found in low concentrations in the whale hypophysis (Geiling, 1935); the factor of postmortem loss of the hormone was difficult to assess. Leblond and Noble (1937), using the local crop response, obtained positive luteotropin tests with extracts from the hypophyses of fish, amphibians and reptiles.

IV. Isolation and Properties of Luteotropin

1. *Extraction from Hypophyseal Tissue and Purification*

The relative stability of the luteotropic hormone to chemical reagents has made it possible to extract this principle from hypophyseal tissue by the use of a variety of procedures. These methods have been applied either to minced, fresh tissue or to acetone-desiccated hypophyseal glands. Three types of extraction media have been employed: 1) alkaline extraction (aqueous, or aqueous alcohol); 2) acid extraction (aqueous, or aqueous acetone), and 3) chloroform extraction. Beef and sheep glands have been used most extensively for the preparation of luteotropin. There are few data on the preparation of this hormone from the hypophyses of other species.

Bergman and Turner (1937) made a careful study of four methods employed frequently for the extraction of the luteotropic hormone. These methods differed in the solvent used for the initial extraction. One employed aqueous, alkaline extraction at low temperatures (Gardner and Turner, 1933); a second was an acid, aqueous-acetone procedure (Lyons and Catchpole, 1933–34); the third involved extraction with glacial acetic acid (McShan and Turner, 1934–35), and the fourth used alkaline, aqueous alcohol (Bates and Riddle, 1935). It was found that an alkaline 60 to 70% ethanol extraction procedure was superior as judged by both total yield of luteotropin and physiological activity per milligram of extracted solids.

Extraction of hypophyseal tissue with alkaline solvents, however, removes not only more of the luteotropic hormone but also of other hypophyseal proteins, both hormonal and nonhormonal. Therefore, while an alkaline extract is exceedingly useful as a means of obtaining a rather complete mixture of the hormones present in the hypophysis, the presence of all of the active principles makes more difficult the separation in pure form of a particular protein hormone. This difficulty is circumvented in either the acid or the chloroform methods of extraction.

Extraction of luteotropin by acid solvents was suggested by Lyons and Catchpole (1933–34), who used acid acetone. At a later date, glacial acetic acid was employed by McShan and Turner (1934–35). The acid-acetone technique has been used by many investigators, since it yields an extract containing significant quantities of luteotropin, and yet is relatively free of other hypophyseal proteins. One major contaminant is the adrenotropic hormone from which separation is readily effected. Thus the same extraction procedure is useful as an initial step in the isolation of adrenotropin. Minced fresh hypophyseal tissue (either dissected adenohypophyseal tissue or whole glands) is extracted with approximately 60 to 80% acetone which has been acidified with concentrated hydrochloric acid. The final pH of the tissue-solvent mixture is between 1.0 and 2.0. Removal of the tissue and raising the acetone concentration of the extract to approximately 90 to 92% results in the precipitation of the crude luteotropin. The procedure was originally described briefly by Lyons and Catchpole (1933–34), and by Lyons (1936–37) and has since been presented in detail (Lyons, 1937; Li *et al.*, 1942; White *et al.*, 1942; White, 1943).

Purification of the crude luteotropin may be achieved in several ways. Extraction with water separates the water-soluble hydrochloride of the hormone from an insoluble residue (White *et al.*, 1942; White, 1943). Addition of acetone to 92% concentration precipitates the luteotropin. At this stage the product is a mixture of several proteins. One is luteotropin; a second protein is the adrenotropic hormone, another is a protein insoluble at pH 6.6, and a fourth component is a protein which is very soluble over the entire pH range. Solution of the crude luteotropin at pH 8.0, and adjustment to pH 6.6 in the presence of 0.05 saturation with ammonium sulfate removes the pH 6.6 insoluble protein. From the supernatant, purified luteotropin may be obtained by adjustment to pH 5.4. Further purification is obtained by repetition of this fractionation procedure. This method of purification (White *et al.*, 1942; White, 1943) yields a product of maximum physiological activity and with physical and chemical properties similar to those found for crystalline prolactin preparations (White *et al.*, 1942; White, 1943) and for purified, amorphous products (Lyons, 1937; Li *et al.*, 1942).

Satisfactory purification of crude prolactin has also been achieved (Li *et al.*, 1942) by taking advantage of the differences in solubility between the luteotropic and adrenotropic hormones at pH 3.0 in the presence of 0.36 molar sodium chloride. Under these conditions, the luteotropic hormone is precipitated, whereas adrenotropin remains in solution. Further purification of luteotropin is achieved by precipitation at its isoelectric point (pH 5.7) and repetition of the pH 3.0-sodium chloride procedure.

Luteotropic preparations with maximum potency have also been obtained by purification of extracts made initially by extraction of fresh, ground hypophyseal tissue with chloroform (Schwenk *et al.*, 1943). This procedure was developed in an attempt to circumvent the troublesome overlapping of hormone fractions encountered in other methods, which made more difficult the preparation of each hormone in pure form. The chloroform technique offered the possibility of fractionating the starting material so that there might be achieved the preparation of several hormones from one batch of glands. Sevag (1934) had successfully used chloroform precipitation of proteins from aqueous solution in the purification of polysaccharides. When hypophyseal tissue was ground and shaken with chloroform at a pH of 5 to 6 and the mixture subsequently centrifuged, three distinct layers were formed. The lowest consisted of chloroform, in which most of the lipide substances of the tissue was present. The layer above the chloroform was a gel which contained the bulk of the tissue proteins, together with luteotropin and adrenotropin. In the top, clear aqueous solution were found thyrotropin, gonadotropins, pituitrin, and other easily soluble substances. The luteotropic hormone was obtained from the chloroform gel by extraction with acid methanol and fractionation with sodium chloride. The yield of purified hormone was approximately ten times that obtained by previously described techniques.

From highly purified luteotropin preparations, a crystalline protein has been obtained (White *et al.*, 1937, 1942; White, 1943) with properties indistinguishable from those of the purified amorphous preparations of the hormone. The procedures described for the crystallization of the protein are apparently not satisfactory since they have not been uniformly reproducible. Although crystallization of luteotropin has been accomplished in one additional laboratory (Schwenk, E., personal communication), lack of success has been reported in another (Bates, W. R., personal communication). Further careful study should lead to a more satisfactory method of preparing not only luteotropin, but also other of the adenohypophyseal hormones, in crystalline form. The recent preparation in high yields of a crystalline protein with marked growth hormone activity is a noteworthy effort in this direction (Fishman *et al.*, 1947, 1948; Wilhelmi *et al.*, 1948). While crystallinity is not in itself an adequate criterion of purity of a protein, crystallization techniques are often useful as an aid in achieving further purification of proteins.

2. Physical and Chemical Properties

Preparations of luteotropin from both beef and sheep glands have been described which meet satisfactorily the criteria at present available for estimating the degree of purity of proteins (Shedlovsky, 1943). The most exacting of these criteria are electrophoretic and ultracentrifugal homogeneity, and satisfactory accord with the Gibbs phase rule in solubility behavior.

The electrophoretic behavior of purified luteotropin preparations from both sheep and beef hypophyseal tissue has been studied by Li *et al.* (1939–40, 1940a, 1942). The electrophoretic properties of crystalline luteotropin from beef hypophysis have been examined in the Tiselius apparatus by Shipley *et al.* (1939) and by White *et al.* (1942). The crystalline preparation has also been studied by the cataphoretic technique (White *et al.*, 1942) based upon measurement of the electrophoretic mobility of microscopically visible quartz particles coated with an absorbed layer of protein. The data obtained show that the luteotropic products from beef and sheep hypophyses are indistinguishable in their electrophoretic behavior and have an isoelectric point at pH 5.7. However, a difference has been observed (Li *et al.*, 1940a, 1940–41) in the solubility behavior of luteotropin prepared from beef glands as compared to the product obtained from sheep hypophyseal tissue, since, at the same pH and salt concentration, the beef luteotropic hormone was less soluble than the preparation from sheep glands. Indeed, it was reported that if solid sheep hormone was added to a saturated solution of the beef hormone, the former went into solution. The greater insolubility of beef luteotropin was attributed in part to its higher tyrosine content since the beef luteotropic product contained 5.7% tyrosine whereas the sheep preparation had 4.5% of this amino acid (Li *et al.*, 1941b). Fleischer (1943) studied the solubility of luteotropin in organic solvents and showed that, at a pH below its isoelectric point, the hormone is highly soluble in 99.8% methanol and in 95% ethanol.

At the present time there is some difference of opinion regarding the molecular weight of the luteotropic hormone. This difference apparently does not depend on the species from which the hormone was prepared. White *et al.* (1942) found a sedimentation constant of 2.8 for luteotropin. Assuming a spherical shape for the molecule, this sedimentation constant indicated an approximate molecular weight of 35,000 for the protein hormone. The same sample of prolactin was examined independently in the laboratory of Professor J. W. Williams, at the University of Wisconsin. The values reported to the writer by Professor Williams were: 2.65 S for the sedimentation constant; 7.5×10^{-7} for the diffusion constant; and 32,000 for the molecular weight (White *et al.*, 1942). On the other hand, osmotic pressure measurements (Li *et al.*, 1941b) have indicated a molecular weight of approximately 26,000, and diffusion (sintered glass membrane) and viscosity measurements assigned a molecular weight of 22,000 to luteotropin (Li, 1942). From the two sets of data, the calculated dissymmetry constants (f/f_0) are 1.37 and 1.29 (Li, 1942). The optical rotation of luteotropin has also been established (Li, 1942). The more important physical chemical properties of luteotropin are compiled in Table I.

The differences which have been reported for the molecular weight of luteotropin have been difficult to reconcile. On the basis of the sulfur and tryptophane content (see below) of the hormone, the calculated minimal molecular weights are in better agreement with the higher than with the lower molecular weight values which have been reported for luteotropin. Recently, Li (personal communication) has stated that, from his extensive analytical study of the composition of sheep luteotropin, a minimum molecular weight of 33,300 is indicated. This would bring several laboratories into harmonious agreement.

TABLE I

*Some Physicochemical Properties of Luteotropin**

Property	Literature cited
Solubility	
In 0.357 *M* NaCl at pH 2.25	
0.506 g./l. (sheep)	Li *et al.* (1940–41)
0.316 g./l. (beef)	Li *et al.* (1940–41)
Isoelectric point	
pH 5.60	Shipley *et al.* (1939)
pH 5.65	White *et al.* (1942)
pH 5.70	Li *et al.* (1939–40)
pH 5.73	Li *et al.* (1940a)
Molecular weight	
From osmotic pressure 26,500	Li *et al.* (1941b)
From diffusion and viscosity 22,000	Li (1942)
From sedimentation—diffusion 32,000	White *et al.* (1942)
Diffusion constant (D_{20}w)	
Sintered glass membrane 9.0×10^{-7}	Li (1942)
Free diffusion 7.5×10^{-7}	White *et al.* (1942)
Partial specific volume (V_1) 0.721	Li (1942)
Viscosity coefficient ($\eta/\eta_0 - 1$)1000/cV.) 6.65	Li (1942)
Dissymmetry constant (f/f_0)	
Viscosity data 1.29	Li (1942)
Sedimentation—diffusion 1.37	Li (1942)
Optical rotation $[\alpha]_{25°}^{Na} = -40.5°$	Li (1942)

* Except for solubility differences between beef and sheep luteotropins, the other physicochemical properties appear to be independent of species source of the hormone.

The ultraviolet absorption spectrum of luteotropin (White and Lavin, 1940) is typical of that found for a number of proteins that are free of nonamino acid residues which absorb in the region of 2500 to 3000 A. (Lavin and Northrop, 1935; Coulter *et al.*, 1935–36). The broad band with a maximum at 2800 A. is evidence for the presence of aromatic amino acids in luteotropin.

The elementary composition of luteotropin is typically that of a protein. Table II contains some of the analyses which have been reported for the elementary composition of the hormone. Qualitative tests for phosphorus, carbohydrate, and sulfhydryl groups are negative. Until very recently, the amino acid composition had not been studied in detail.

The cystine content has been reported as 3.0 (Li *et al.*, 1941b; Fraenkel-Conrat, 1942), 3.4 (White *et al.*, 1942) and 3.1% (Li, 1943) and a value of 4.3% was found for methionine (Li, 1943). Within the limits of experimental error, the cystine and methionine contents of both beef and sheep luteotropic hormones are the same and account for the total sulfur in the preparations. Other amino acid values for beef luteotropin have been reported: arginine, 8.3% (Li *et al.*, 1941b); tryptophan 1.3% (Li *et al.*, 1940b; White *et al.*, 1942), and 2.5% (Li *et al.*, 1941b); tyrosine, 5.5% (White *et al.*, 1942) and 5.7% (Li *et al.*, 1940b, 1941b). The values obtained on analysis of the hormone from sheep glands were similar, with the exception of that for tyrosine, which was found to be 4.5% (Li *et al.*, 1940b, 1941b). The variation in tyrosine content of the hormones from the two species, and the difference in solubility, mentioned previously, are the only dissimilarities which have as yet been established in comparisons of the beef and sheep luteotropic hormones. Despite these detectable physical and chemical differences, the hormones from beef and sheep glands are immunologically indistinguishable (Bischoff and Lyons, 1939).

TABLE II

*Elementary Composition of Various Luteotropin Preparations**

Preparation	C	H	N	S	Ash
	%	%	%	%	%
Purified luteotropin	52.04	7.01	16.84	2.05	0.59
Crystalline luteotropin	51.81	6.81	16.49	2.03	0.50
Luteotropin (Schwenk)	51.10	7.00	16.61	1.98	0.72
Luteotropin (Lyons)	51.40	7.01	16.09	2.24	Negligible
White, Catchpole, and Long (1937)	51.11	6.76	14.38	1.77	Negligible
Li, Lyons, and Evans (1941b)				1.79	

* After White, A., *Ann. N. Y. Acad. Sci.* **43**, 341 (1943).

3. Amino Acid Composition

There is good agreement among the data for the amino acid composition of the luteotropic hormones prepared in different laboratories, with the exception of the tryptophan values. A tryptophan content of 1.3% was obtained when analysis was conducted on completely hydrolyzed luteotropin (Li *et al.*, 1940b; White *et al.*, 1942); a higher value of 2.5% was found (Li *et al.*, 1941b) by a glyoxylic method (Shaw and McFarlane, 1940) applied to the intact protein. Brand (Brand, E., private communication) has confirmed the lower tryptophan value by finding 1.2% of this amino acid in a preparation of luteotropin supplied by the writer. The procedure employed in Brand's laboratory was a photometric technique

TABLE III

*Some Amino Acid Analyses of Luteotropin**

Amino acid	Per cent	Literature cited
Tyrosine		
Beef luteotropin	5.5	White *et al.* (1942)
	5.7	Li *et al.* (1940b, 1941b)
Sheep luteotropin	4.5	Li *et al.* (1940b, 1941b)
Tryptophan	1.2	Brand (Brand, E., private communication)
	1.3	Li *et al.* (1940b); White *et al.* (1942)
	2.5	Li *et al.* (1941b)
Cystine	3.0	Fraenkel-Conrat (1942); Li *et al.* (1940b)
	3.1	Li (1943)
	3.4	White *et al.* (1942)
Methionine	4.3	Li (1943)

* Except for indicated differences in tyrosine contents, beef and sheep luteotropins appear to have the same percentages of the other amino acids which have been studied.

TABLE IV

Composition of Lactogenic Hormone

(Mol. Wt. = 33,300)

(Unpublished data of Li, C. H., and Lewis, J. C.)

Constituent	Grams per 100 g. protein	N as per cent of protein N	Estimated number of residues
Nitrogen	15.86		
Sulfur	1.79		
Amide N	1.0	6.3	24
Arginine	8.6	17.4	17
Aspartic Acid	11.6	7.6	28
Cystine	3.1	2.3	4
Glutamic Acid	14.1	8.5	32
Glycine	4.0	4.7	18
Histidine	4.5	7.7	10
Isoleucine	6.5	4.4	17
Leucine	12.5	8.4	32
Lysine	5.3	6.4	12
Methionine	5.0	3.0	11
Phenylalanine	4.1	2.2	8
Proline	6.2	4.8	18
Serine	6.5	5.5	21
Threonine	4.1	3.0	11
Tyrosine	4.7	2.3	9
Tryptophan	1.2	1.0	2
Valine	4.8	3.6	14
	Total	99.1	288

(Brand and Kassell, 1939) based on the method described by Lugg (1938). The data in the literature for amino acid analyses of luteotropin are assembled in Table III.

Inasmuch as the writer was aware that Dr. C. H. Li, of the University of California at Berkeley, was actively investigating the amino acid composition of the luteotropic hormone, he wrote to Dr. Li for possible further data, which might be included in this review. Recently, just prior to the completion of this manuscript, Dr. Li generously supplied the extensive data assembled in Table IV. The analyses present, for the first time, a complete picture of the amino acid composition of the lactogenic hormone (luteotropin) from sheep hypophyseal tissue. Dr. Li comments that the tryptophan value has been arrived at as a result of many colorimetric and microbiological assays; the figure agrees with the lower value in the literature (Table III). The methionine and tyrosine values now presented by Dr. Li are somewhat higher than the values reported previously (compare Tables III and IV). The complete analyses represent an important contribution to our knowledge of the protein hormones of the hypophysis.

4. Effect of Physical and Chemical Treatment on Biological Activity of Luteotropin

a. Heat and pH. Earlier studies (Riddle and Bates, 1939) of the heat lability of luteotropin suggested that the hormone was relatively thermostable when heated in a boiling water bath for 1 hour at pH 7.0 to 8.0, but less stable at other pH values. However, the reported relative heat stability of the luteotropic hormone, as is the case of most other biocatalytically active proteins, may be influenced by a variety of factors. The presence of other proteins may result in an increased resistance of each of the proteins in the mixture toward labilizing agents. Moreover, the concentration of protein and inorganic electrolyte and the pH of the solutions examined may influence the nature of the data obtained. Experiments with purified luteotropin (White *et al.*, 1942) indicated that this hormone may be classed as a heat-labile substance. Destruction of hormonal activity by heat proceeded most rapidly in solutions more alkaline than pH 11.0, and exposure of a 0.04% solution of luteotropin to 100°C. for 30 minutes resulted in considerable loss of biological activity over the entire pH range. In alkaline solution, this heat lability is accompanied by a splitting of labile sulfur from the hormone; a significant degree of hydrolysis of the protein occurs at elevated temperatures, in either acid or alkaline medium.

b. Hydrolysis. The hydrolysis of luteotropin with boiling 20% hydrochloric acid gave (White *et al.*, 1942) an hydrolyzate which retained no evidence of lactogenic activity even when assayed at levels approximately 400 times that required to produce a physiological response with the unhydrolyzed protein. Hydrolysis with pepsin or trypsin also resulted in rapid inactivation of the hormone (McShan and French, 1937; White *et al.*, 1942). The data of White *et al.* (1942) suggested that destruction of physiological activity occurred prior to hydrolysis of the protein into fragments which were no longer precipitable by trichloroacetic acid.

c. Denaturing Agents. Li *et al.* (1941a, b) and Li (1942, 1944) have studied the effects of urea and of a commercial detergent on the properties of the luteotropic hormone. Using viscosity measurements as an index of alterations in the physical state of the protein, it was demonstrated that, in the presence of 3.0 M urea, the relative viscosity of the luteotropic hormone is greatly increased. However, osmotic pressure studies revealed that the hormone does not dissociate in 6.66 M urea solution. Unfortunately, it was not possible to determine the biological activity of the hormone in urea solutions, but after removal of the urea by dialysis, the physiological activity was found to be the same as that noted before urea treatment. In the case of urea, therefore, it was not possible to establish unequivocally whether the denaturing agent also affected physiological activity. Similar increases in viscosity of solutions of the protein were also produced with solutions of detergents; a distinct lowering of the biological activity of the hormone resulted. The reversibility of these alterations could not be assessed, since efforts to remove the detergent from the protein were unsuccessful. Inasmuch as the biological activity of luteotropin appears to depend in part on the presence of intact phenolic hydroxyl and unsubstituted amino groups in the molecule (see below), the loss of hormonal activity in the detergent studies might have been due to interactions between the detergent and either or both of these groups.

d. Acetylation. Ketene has been employed widely as a reagent for examining the effect of substitution of the amino groups and the phenolic hydroxyl groups of tyrosine on the activity of proteins which exhibit one or another type of biocatalytic or physiological behavior. Acetylation of proteins with ketene has the advantage that the reaction may be conducted under conditions (aqueous solutions and low temperatures) which do not alter appreciably the native state of the protein. It has been assumed generally that ketene effects a degree of selective acetylation in the case of most proteins, since amino groups have been reported to react much more rapidly than do hydroxyl groups (Bergmann and Stern, 1930; Herriott and Northrop, 1934–35; Herriott, 1935–36; Stern and White, 1937–38). However, it should be emphasized that investigations of the reaction of proteins with ketene have concentrated attention on measurements of the degree to which free amino or phenolic hydroxyl groups have been substituted, and have ignored the possible acetylation of aliphatic hydroxyl groups present in certain amino acids, *i.e.*, serine and threonine. Inasmuch as it is unknown at the present time whether these two amino acids have a role in determining the biocatalytic activity of certain proteins, the results obtained by the use of ketene as an acetylating agent, and by the measurement of the degree of substitution of amino and phenolic hydroxyl groups, are not possible of interpretation solely in terms of the contribution of these groups to the activity of the protein. Although data and conclusions obtained from studies of the inactivation of luteotropin by ketene will be presented, in no single case has the action of ketene been shown to be limited only to those protein groupings whose biological importance is being assessed.

In a preliminary report, Li *et al.* (1939a) observed that the treatment of luteotropin with ketene at 20°C. for 5 minutes resulted in acetylation of all the free amino groups of the hormone and a complete loss of physiological activity. It was not demonstrated that substitution of groups other than free amino groups had not occurred. These additional data seemed necessary, since proteins may differ in the rates at which different groups in the molecule may react with ketene. Indeed, in a later complete publication, Li and Kalman (1946) observed that the reaction of luteotropin with ketene proceeded in an unexpected manner. Acetylation of up to 20% of the phenolic hydroxyl groups of tyrosine residues in the protein was reported to have been achieved by treatment with ketene for 3 minutes at pH 7.0 in phosphate buffer. Under these experimental conditions, no acetylation of free amino groups was observed. The product obtained exhibited the full biological activity of the original luteotropin. Under these conditions, therefore, ketene appeared to react with the phenolic groups of tyrosine residues in the hormone molecule more rapidly than with the amino groups. On the other hand, in acetate buffer at pH 4.0, the extent of acetylation of both amino and phenolic groups after 5 minutes of treatment was practically identical, 35% of each type of grouping being acetylated. This product had only one-fifteenth the biological activity of the unacetylated hormone. In other preparations in which 70% of the amino groups and 70% of the phenolic hydroxyl groups were acetylated, the biological activity was reduced to almost the vanishing point. The authors concluded that the decrease in crop stimulating potency of the acetylated products was due to a substitution of the free amino groups of the protein hormone. This conclusion that the amino groups are essential for the biological activity of the hormone was given further support by the demonstration that hydrolysis of the phenolic oxygen-acetyl groups in the acetylated, inactive protein under conditions known not to inactivate the luteotropic hormone (pH 11 for 10 minutes) did not restore the biological activity. This would be the expected result if the decreased potency of acetylated luteotropin preparations was unrelated to the degree of involvement of the tyrosine residues of the molecule, but dependent solely on the free amino groups.

e. Other Reactions Involving Free Amino Groups. Luteotropin has been treated with a number of reagents which affect chiefly the free amino groups of proteins. However, in the case of each of these reagents, adequate data have not been presented to establish that only free amino groups have been altered by the reagent employed. The data, therefore, must again be interpreted with reservation. Hormonal activity was destroyed by treatment with sodium nitrite, formaldehyde, benzoyl chloride, or by diazotization (McShan and French, 1937). Groups other than free amino groups were probably involved in these reactions. Treatment with nitrous acid at low temperatures also resulted in complete inactivation of the hormone (Li *et al.*, 1939b). Even at low temperatures, however, alterations other than deamination of the free amino groups occur when proteins react with nitrous acid (Wiley and Lewis, 1930).

Treatment of luteotropin with phenylisocyanate at low temperatures yielded a phenylureido derivative which had only about 10% of the biological activity of the untreated hormone (Bottomley and Folley, 1940). The specificity of phenylisocyanate as a reagent for only the free amino groups of proteins has been seriously questioned (Miller and Stanley, 1941).

f. Iodination. The effect of iodination on the biocatalytic activity of various proteins has been employed as an approach to determining the contribution which the tyrosine residues of the protein make to the specific activity. However, these studies have not given adequate consideration to other types of reactions which may also occur when proteins are treated with iodine. Changes in the protein of an oxidative nature may ensue, as may the iodination of protein groupings (Bauer and Strauss, 1935) other than the aromatic nuclei of the tyrosine residues. The reaction of luteotropin with iodine has been studied in some detail by Li *et al.* (1941a). Iodination in phosphate buffer of pH 7.0 at 20°C. for 1 hour resulted in an introduction of iodine into the phenolic portions of the tyrosine residues of the protein, with an accompanying complete loss of hormonal activity.

g. Effect of Thiol Compounds. The reducing action of thiol compounds on luteotropin produced a varying degree of inactivation, depending on the extent of reduction (Fraenkel-Conrat *et al.*, 1942). Treatment of a solution of luteotropin with a forty-fold concentration of cysteine transformed the hormone into an insoluble protein which, when redissolved under conditions which prevented autoxidation, was as active biologically as the untreated hormone. However, when a two hundredfold quantity of cysteine was employed to reduce luteotropin, inactivation occurred. Thioglycolic acid was approximately fifty times more effective than cysteine in causing inactivation of the hormone.

h. Esterification. An effort has been made to assess the contribution of the carboxyl groups of luteotropin to the biological activity of the hormone. Progressive esterification of luteotropin with methyl alcohol (Li and Fraenkel-Conrat, 1947) caused a gradually increasing degree of loss of potency. Electrophoretic studies suggested that the esterified hormone was essentially homogeneous and, as expected, more basic. The conclusion was drawn that carboxyl groups are necessary for the biological activity of luteotropin, although the possible involvement of other groups is not ruled out by the esterification procedure employed.

In summarizing the physical and chemical data on luteotropin (White, 1946), it is evident that the purity of the hormone has been established by satisfying the criteria usually employed in studies of protein purity. The physicochemical properties of luteotropin have been well characterized. There is no evidence for the presence in the molecule of residues other than amino acids, and recent amino acid analyses account practically quantitatively for the total nitrogen of the protein. The hormone is a heat-labile protein, rapidly inactivated by mild hydrolysis or by reagents which have been assumed to affect chiefly the free amino, or disulfide groups of the protein. Although acetylation of the phenolic hydroxyl groups of the tyrosine residues has been reported not to diminish the biological activity of luteotropin, iodination of these residues was accompanied by a rapid destruction of biological potency.

V. Physiological Aspects of Luteotropin

Most of the investigations which have been published dealing with the physiology of adenohypophyseal luteotropin are difficult to evaluate for several reasons. In the first place, many of the investigations were conducted at a time prior to the isolation of this hormone in highly purified form, free from other known hypophyseal principles. As a consequence, it is frequently not possible to judge which of the reported physiological responses, seen as a result of the administration of a particular preparation, are due to luteotropin, *per se,* and which may be a reflection of the simultaneous action of several hormones. Secondly, a large portion of the studies of the physiological behavior of luteotropin have been conducted in the dove or pigeon; the others in mammals. The data obtained suggest that this hormone may elicit rather widely different physiological responses in the bird, as compared to the mammal,

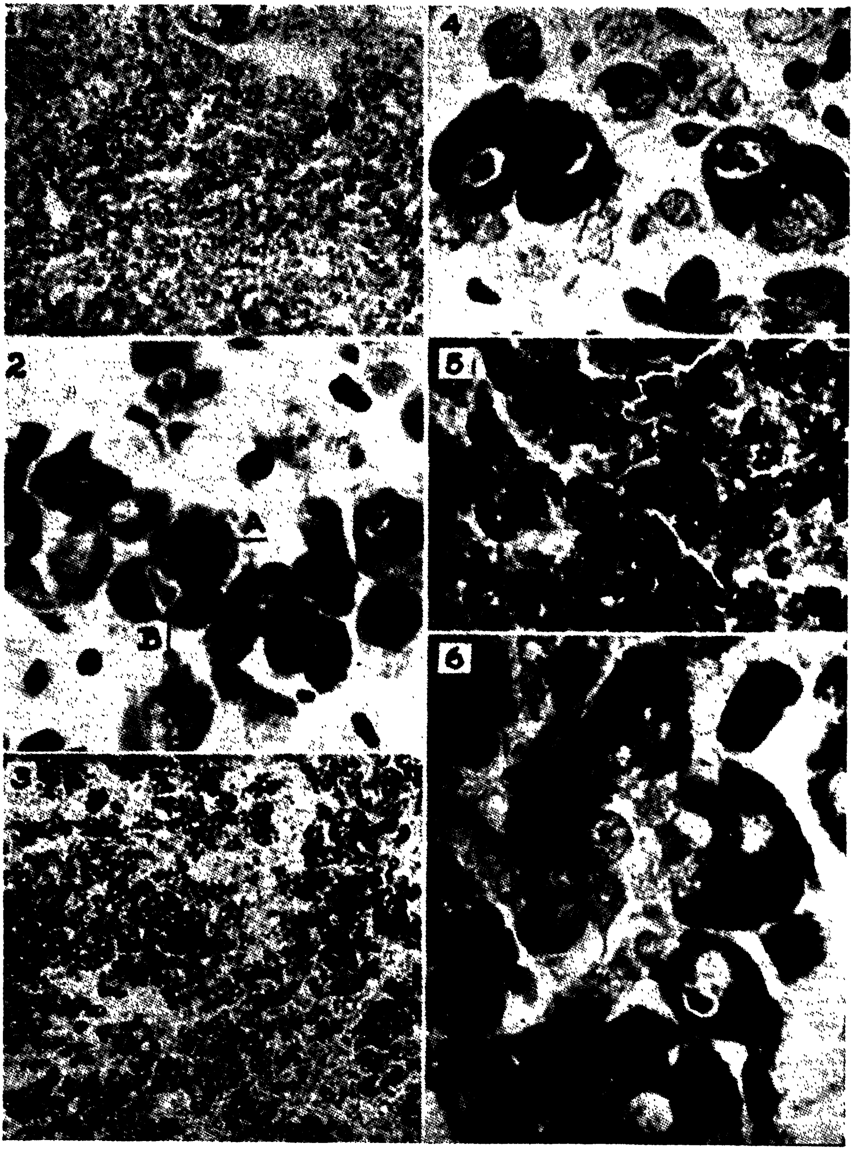

FIG. 6, 1. An area from the adenohypophysis of a rabbit in estrus, showing the characteristic distribution of the carmine cells (black) in an unstimulated gland. Formalin fixation; Heidenhain's azan modification of Mallory. ×120.

2. A small cluster of carmine cells (dark) with a few orange acidophiles (gray) from an unstimulated gland, showing the relative size of the two types of cells and the differences in the character of their granulation. A lightly-stained carmine cell is

although the extent to which these differences are real needs to be reassessed with the purer hormone preparations now available.

1. Site of Secretion in the Adenohypophysis

The majority of the studies in the literature, which have attempted to correlate the sequence of cytological and physiological events in the adenohypophysis, have made use of the classical division of cell types into acidophiles, basophiles, and chromophobes. However, a discussion of the secretion of luteotropin, as well as of the other gonadotropins, necessitates consideration of a particular type of acidophile, viz., the carmine cell discovered by Dawson and Friedgood (1938). This cell was described in studies which directly traced the origin of metakentrin (LH) to the acidophiles by correlating the sequence of cytological and physiological events in the adenohypophysis with the characteristic effects of this hormone on the mature ovarian follicles of the cat and rabbit (Friedgood and Dawson, 1938, 1940, 1942). The hypophyses were subjected to formol-sublimate fixation, followed by differential staining with Heidenhain's azan modification of Mallory's connective tissue stain (Dawson and Friedgood, 1938). Although the morphological appearance of the acidophile that secretes metakentrin, the luteinizing hormone, differs remarkably from the ordinary acidophiles, it is not a new type of cell except insofar as it represents a functional phase of the secretory cycle of the acidophiles. Under the conditions of histological preparation mentioned above, this cell takes a deep, red stain whereas the ordinary acidophiles are orange; the basophiles are deep or light blue, and the

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shown in mitosis (arrow A). Note the distinct macula (arrow B) in three carmine cells. Formol-sublimate fixation; Heidenhain's azan modification of Mallory. ×1000.

3. An area from the adenohypophysis of a rabbit one and one-half hours after mating, showing the increase in size and number of the carmine cells and the general pattern of their distribution (compare with A, above). Formol-sublimate fixation; Heidenhain's azan modification of Mallory. ×120.

4. An area from the adenohypophysis of a rabbit in estrus, showing the occasional large, coarsely granulated carmine cells (black) which occur in association with smaller, more finely granulated acidophile cells (gray). Formalin fixation; Heidenhain's azan modification of Mallory. ×1000.

5. An area from the same gland as in C^3, above, showing a dense aggregation of carmine cells with scattered orange acidophiles. Formol-sublimate fixation; Heidenhain's azan modification of Mallory. ×240.

6. An area from an activated gland showing densely stained carmine cells (black) and orange acidophiles (gray). Formol-sublimate fixation; Heidenhain's azan modification of Mallory. ×1000. (After Friedgood, H. B., and Dawson, A. B. 1938. *Endocrinology* **22**, 674.)

chromophobes are light pink to colorless. Because the cell contains large, coarse, irregular granules with a striking affinity for the azocarmine dye, which is extracted readily from the ordinary acidophile, it was named the "carmine cell." More recently, Dawson (1946) has referred to this cell as the "carminophile." Figure 6, 1–6, illustrates some of the morphological and staining characteristics of this cell.

On the basis of their observations, Friedgood and Dawson (1942) concluded that: (a) the carmine cell reaction constitutes histological evidence of the enhanced gonadotropic activity of the adenohypophysis which results from coitus, (b) that the carmine cell contains a luteinizing hormone, which is essential for the initial maturation and subsequent ovulation of the ovarian follicles, and (c) that degranulation of the cell represents the secretory phase during which, if the extent of the carmine cell reaction is sufficient and the degranulation adequate, enough hormone is released to initiate maturation and to induce ovulation. Although their data indicated that in the postcoital period the carmine cells are related to elaboration and secretion of the luteinizing hormone, Friedgood and Dawson (1942) suggested that these cells may be associated also in some way with other gonadotropic functions of the adenophyophysis since they are present in extraordinarily large numbers in the cat's hypophysis during the last week of pregnancy and *early lactation.* The total evidence available regarding the relation of the carmine cell to the gonadotropic functions of the adenohypophysis, together with the demonstration that the luteotropic hormone, then termed prolactin, maintains a functioning corpus luteum (see below), led Friedgood (1946) to state that "circumstantial evidence indicates that prolactin is derived from a cell with identical morphological characteristics" (i.e., as the carmine cell).

The validity of this conclusion that the luteotropic hormone is secreted by the carmine cell of the adenohypophysis is supported by the data in the literature. Schooley and Riddle (1938) studied the histology of the pigeon's adenohypophysis and observed that, during the first week of incubation, the acidophiles increased in number to the point where sparsely granulated cells predominated. This condition was maintained until the end of feeding of the young. Friedman and Hall (1941) found that luteotropin was more concentrated in the acidophilic zone of the beef hypophysis. Desclin (1945) presented further direct evidence of the production of luteotropin by the acidophiles in confirmation of the observations of Friedgood and Dawson (1942). This investigator found that immediately after parturition in the rat, lactation caused an intense degranulation of acidophiles in the hypophysis. Actual counts revealed that the number of acidophiles decreased from 32% of the total cell number on the fifth day postpartum without lactation, to 13% on the twentieth day of lactation. The acidophilic picture was the same in castrated, lactating females, thus ruling out ovarian influences. The basophiles showed changes, but to a lesser degree, in normal lactating rats, but were almost unchanged in the castrate series. In other experiments (Desclin, 1945), lactation was prolonged for 30 days by substituting younger litters on the fifteenth day of lactation. In all instances, the degranulation of the acidophiles and the reduction in their number was correlated directly with the initiation and maintenance of lactation.

Similar data were presented at the same time by Everett and Baker (1945), who also studied the distribution of cell types in the adenohypophysis of the rat during late pregnancy and lactation. The acidophiles were greatly increased in early lactation, rising from 19% in late pregnancy (twentieth day) to a maximum of 36% on the third day of lactation and gradually decreasing in number to reach a level of 29% on the twenty-second day. A correlation was established between the increase in number of acidophiles, with their increased granulation during early lactation, and the high luteotropin content of the adenohypophysis during that period. A year later, Dawson (1946) reported a direct study of the effects of nursing and lactation on the carminophiles of the adenohypophysis of the cat. The effects of nursing were apparent as early as 24 hours after birth of kittens. The carminophiles became elongated and more definitely oriented toward the blood vessels, with initial degranulation occurring. Earlier, there were seen cycles of carminophilic activity in the preimplantation period and in late pregnancy through lactation. These cycles were directly related to luteal function and to lactation. It is of considerable interest that Dawson (1946) also recorded the observation that nervous stimuli (mechanical stimulation of the uterine cervix at mating and mechanical stimulation of the nipples by suckling young) resulted in rapid elaboration and release of secretory products by the carminophiles. Folley (1940) has reviewed the role of nervous influences in lactation. These data, together with those discussed above, permit the conclusion that carmine cell activity is correlated closely with the initiation and maintenance of lactation, and therefore, it may be concluded that the carmine cell of the adenohypophysis is the site of elaboration and secretion of luteotropin.

2. Factors Influencing the Luteotropin Content of the Hypophysis

Bates *et al.* (1935) found, per gram of fresh tissue, nearly equal amounts of luteotropin in the adenohypophyseal tissue of veal calves, adult steers, adult bulls and nonpregnant cows. Larger amounts of hormone were found in the glands of pregnant cows. It is of interest that luteotropin is apparently present in higher concentration in the hypophyses from broody hens than in those from laying hens or males (Burrows and Byerly, 1936). Also, hypophyseal tissue from laying hens and from males of broody genetic constitution contained more luteotropin than those from hens and males, respectively, of nonbroody genetic constitution (Byerly and Burrows, 1936). The role of the luteotropic hormone in inducing broodiness in birds will be considered later in this review.

Turner and his associates have been particularly active in determining the influence of a wide variety of physiological conditions on the luteotropin content of the hypophysis. The administration of estrone was found to increase hypophyseal size in both male and female rats (Reece and Turner, 1936a). Since the hormonal concentration per milligram of gland remained the same (Reece and Turner, 1936b), the estrone-treated animals had hypophyses with an increased total content of luteotropic hormone. Conversely, ovariectomy in the mature rat was found to diminish the amount of hypophyseal luteotropin (Reece and Turner, 1937b). In contrast to the rat, the size and luteotropic hormone content of the pigeon's hypophysis were found to be unaffected by the injection of estrone, diethylstilbestrol, progesterone, or testosterone propionate into birds of either sex (Meites and Turner, 1947a). It would appear that the pigeon hypophysis, unlike that of the mammal, is refractory to administration of sex hormones and is not stimulated by the latter to increase its secretion of luteotropic hormone.

Additional differences among species were observed in studies of the effect of pregnancy and nursing on the luteotropin content of the hypophysis. Whereas suckling decreased the hypophyseal concentration of hormone in postpartum rats (Reece and Turner, 1936c), the luteotropin content of the hypophyses of postpartum rabbits was much less in nonsuckling than in suckling does (Meites and Turner, 1942). However, in all species examined, namely, rat, guinea pig, rabbit, and mouse, there is, with the exception of the mouse, a similar general trend in the luteotropin concentration of the hypophysis during pregnancy and postpartum. The hormone content of the gland remained low during pregnancy, and then rose from two- to fourfold shortly after parturition. Soon following this postpartum increase there is a sharp decline in the luteotropic hormone content of hypophyseal tissue of suckled as well as of nonsuckled animals. In the mouse, on the other hand, by the tenth day of pregnancy the luteotropin content of the hypophysis had increased as much as 88% in comparison with values obtained with mature, nonpregnant females (Hurst and Turner, 1942). An additional increase occurred following parturition, with a peak of more than 200% above the level for the virgin female being reached by the fifth day postpartum. Thus, the mouse differs particularly from the rat, guinea pig, and rabbit in its high hypophyseal content of luteotropin during pregnancy.

Depressed thyroid function, induced by thiouracil administration, was reported by Meites and Turner (1947b) to reduce below normal the luteotropin content of the hypophyses of young female rats. Administration of the drug together with estrogens did not impair the ability of the latter hormones to increase the hypophyseal concentration of luteotropin, as reported previously. However, if thiouracil was given for a two-week preliminary period, subsequent administration of estrogen together with thiouracil for 10 days failed to maintain even the normal level of luteotropic hormone in the hypophysis.

3. *Immunological Aspects; Antibody Formation Against Luteotropin*

The protein nature of the adenohypophyseal hormones has been the basis of the production of antibodies to these principles, a phenomenon first described by Collip and his associates (Collip, 1934) as the formation of "antihormones" following the injection of crude hypophyseal extracts. It seems clear at the present time that the so-called "antihormones" are true immune or antibody globulins, which function to neutralize administered hormone and thus to attenuate or to nullify its physiological effectiveness. Young (1938) administered large doses of purified ox luteotropin daily to rabbits and monkeys. The sera of animals injected for periods of 12 weeks or more showed marked ability to prevent the hypertrophic response of the pigeon crop gland to luteotropin. There was some indication that the injection of this antiserum into lactating mice produced a partial inhibition of milk secretion.

Bischoff and Lyons (1939) studied the immunological properties of luteotropic preparations made from beef or sheep hypophyses and found the products to be antigenically indistinguishable as far as could be determined by the techniques employed for active and passive anaphylaxis, the Dale and Arthus reactions, and precipitin and complement fixation tests. Moreover, the crop-stimulating effect of beef and sheep luteotropin in pigeons could be neutralized by injecting the birds with sufficiently large amounts of antiluteotropin serum, prepared in rabbits against either beef or sheep luteotropin. It is of interest that the sensitive immunological methods could not detect differences between these two preparations, despite the evidence discussed previously that the luteotropin from beef glands differs from that derived from sheep tissue both in solubility and in tyrosine content. Chase (Chase, J. H., personal communication) has also demonstrated the appearance of specific, precipitable antibody in the sera of CBA mice of the Strong strain, receiving daily, subcutaneous injections of 1 mg. of highly purified luteotropin (beef) for 18 weeks.

4. Crop-sac Effects

The ability of luteotropin to cause crop-sac thickening in the pigeon and dove has already been touched upon in connection with methods for the assay of this hormone, and has been illustrated in Fig. 1. The first demonstration of an hypophyseal factor concerned with the growth of the crop-sac of pigeons was that of Riddle and Braucher (1931). The hormone was identified by Riddle *et al.* (1932a, 1932b, 1933) and given the name prolactin. These investigators established further that the hormone was effective in a completely hypophysectomized pigeon (cf. also Schooley *et al.*, 1937), as well as in castrated (Riddle and Dykshorn, 1932), in adrenalectomized, and in thyroidectomized birds (Schooley *et al.*, 1937). The data all support the conclusion that the effect of luteotropin in stimulating the growth of the pigeon crop-sac is a primary and direct one. The thickened mucosa may be desquamated intermittently in thick layers of caseous "crop-milk" as a result of either prolonged stimulation by the hormone or by several injections of large amounts of an active preparation.

5. Mammary Gland Effects

The response of the mammary gland to the administration of luteotropin has been considered in relation to the adaptation of this response to a method of assay of the hormone in either the pseudopregnant rabbit or the hysterectomized guinea pig. Within recent years it has become evident that luteotropin will not only initiate lactation in the mammary gland which has been hypertrophied previously by estrogens, but that the hypophyseal hormone can cause mammary hypertrophy when given in conjunction with small doses of estrogen, and may by itself cause thickening of the mammary duct system.

a. Proliferation. The concept that the adenohypophysis exerts an important influence on the growth and development of the mammary gland had been generally accepted since Reece *et al.* (1936) reported that the mammary glands of completely hypophysectomized rats did not respond to the administration of ovarian hormones. This suggestion for a role of the adenohypophysis in the process of proliferation of mammary gland tissue was further substantiated by the effectiveness of hypophyseal replacement therapy in hypophysectomized animals. The administration of crude anterior pituitary extracts, either alone or with ovarian substance, to hypophysectomized animals of several species permitted normal growth and development of mammary glands. The conclusion is warranted that crude anterior pituitary extracts exert a profound influence on mammary growth (Turner, 1939).

Lyons and Catchpole (1933–34) reported that the mammary gland in a virgin female rabbit, normally matured and then castrated, will respond by growth of ducts and alveoli and by lactation to administration of hypophyseal extracts containing luteotropin. These investigators concluded that if significant amounts of ovarian hormones remained in the organism after castration, these hormones could act only by synergistic stimulation with the luteotropic hormone. Later, Lyons (1936, 1937) reiterated his view that the luteotropic hormone could induce alveolar hyperplasia by demonstrating secretion and alveolar proliferation following injection of luteotropin into young male rabbits previously treated for a period of 3 weeks with daily injections of estrogen. Moreover, the injection of luteotropin into young, normal female rats produced excellent mammary development (Lyons *et al.*, 1941), which was attributed to a synergistic action with estrogen and progesterone. These observations were confirmed in other laboratories (Gardner and White, 1941; Leonard, 1943; Smithcors and Leonard, 1943; Reece and Leathem, 1945).

Particularly striking evidence that luteotropin is a mammary growth-promoting hormone was obtained in experiments by Lyons (1942). This investigator prepared the mammary glands of 30 ovariectomized, virgin rabbits by injection of the animals over a period of 4 weeks with estrone and progesterone. Three days after the last injection of these hormones, five different levels of luteotropic hormone were tested in groups of six rabbits. The hormone was given intraductally in such a way that an individual sector in each animal received a constant volume of fluid. Histological study of the glands was valuable because comparison could be made in a single section between lactating and nonlactating lobules from adjoining sectors. It was thus possible to contrast the prolactational growth induced by the estrone and progesterone treatment, and the additional lactational growth induced by luteotropin. Under the influence of the latter hormone, the number and size of epitheleal cells forming the circumference of alveoli was significantly greater than that of the control alveoli; a large number of the alveolar cells were cast off in the formation of the first milk and were replaced by newly proliferating cells, and mitotic figures were observed in the alveolar epithelium during the lactational growth phase. Study of the sections in the regressive stages showed that the decrease in the number and size of the secretory cells after the withdrawal of the luteotropic influence was just as striking as their increase when hormone was administered. Finally, it may be noted that Hooker and Williams (1941) succeeded in retarding the mammary involution in females, isolated from their young, by the administration of luteotropin. The inhibition of involution was manifested chiefly as a maintenance of mammary lobule-aveolar and duct systems in a condition very similar to that at parturition.

The data indicate that luteotropin does have a proliferative effect on the mammary gland of the intact animal. Administration of luteotropin to hypophysectomized animals, together with amounts of estrogen inadequate to cause mammary proliferation, will lead to mammary gland growth, although the luteotropic hormone alone may not produce this result. Luteotropin given alone to hypophysectomized-castrated rats was reported to cause thickening of the duct system (Reece and Leathem, 1945).

A detailed review of the role of the adenohypophyseal hormones, as well as other hormones in the control of mammary gland growth, has been presented recently by Folley and Malpress (1948).

b. Lactation. It will be recalled that the first suggestion for the existence of a specific hormone of the hypophysis essential for the initiation and maintenance of lactation was made by Stricker and Grueter (1928) as a result of their production of lactation in hypertrophied mammary glands of rabbits given injections of crude adenohypophyseal extracts. Since that initial observation, the lactation response has been adapted, as discussed previously, for the assay of the luteotropic hormone. Also, as might be expected, the induction of lactation by adenohypophyseal extracts has been studied extensively from the practical standpoint in the dairy industry. In addition to the cruder extracts employed initially, more purified preparations of luteotropin, as well as other hormones, notably thyroid substance, have been examined in detail in cattle (Folley, 1940; Riddle, 1940; Hooker, 1946; van Wagenen, 1947). There has also been a degree of clinical trial in the human, as the purified luteotropin became available (Riddle, 1940; Winson, 1943).

The evidence that the action of luteotropin in the initiation of lactation in hypertrophied mammary tissue is a direct and primary one was provided by the demonstration (Lyons, 1942) that the injection of the hormone intraductally into hypertrophied mammary glands elicits lactation in the localized sector. More recently, Meites and Turner (1947c) have presented further evidence for the specificity of the luteotropic hormone in the initiation of lactation. Injection of small amounts of luteotropin intraductally into localized sections of the rabbit mammary gland induced lactation in pregnant and pseudopregnant rabbits. On the other hand, similar injections of either thyrotropin, thyroxine or adrenal cortical extract did not produce a lactation response, again emphasizing the specificity of the luteotropin effect. Fig. 7 illustrates the local lactation response obtained by Meites and Turner (1947c) with a preparation of luteotropin (lactogen), as contrasted with the negative effects of thyrotropin, thyroxine and adrenal cortical extract.

A large amount of literature has been published on the question of whether luteotropin is *the* lactation hormone, or whether other endocrine glands have important roles in the initiation and maintenance of a normal or an augmented degree of milk production (see Turner, 1939; Folley, 1940; Folley and Young, 1940; Peterson, 1944; Folley and Malpress, 1948 (Ch. 16)). The adrenal cortex and the thyroid particularly have been implicated in the phenomena attending lactation, and the data have led to the logical conclusion that there is no single "lactation hormone." It is true that Meites and Turner (1947c) found local injections of hormones other than luteotropin to be ineffective in inducing lactation. However, inasmuch as the normal production of milk is a composite metabolic result of the integration of a wide variety of physiological processes whose rates are influenced by endocrine secretions, it need not be surprising that a single hormone cannot reproduce the normal response (sustained lactation) in its entirety or indefinitely. Maintenance of a normal metabolic rate by thyroid secretion, and of normal water, electrolyte, protein, and carbohydrate regulatory influences by adrenal cortical hormones, would appear to be obvious physiological *sine qua non* for continued lactation. This does not, however, decrease the significance of the observations of Lyons (1942) and of Meites and Turner (1947c),

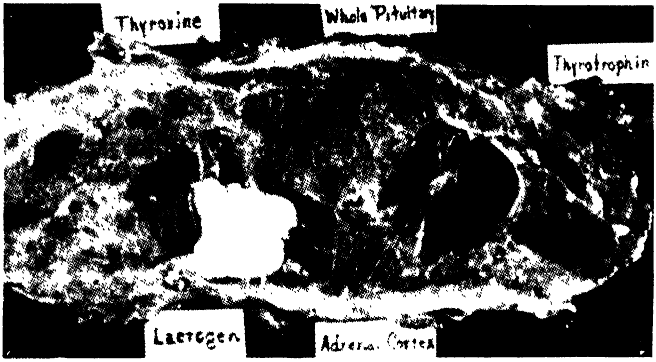

FIG. 7. Effect of different hormones injected intraductally into individual teats of a pseudopregnant rabbit. The whole mount of the mammary glands shows the local lactational response induced with lactogen (luteotropin). The lesser response obtained with whole hypophyseal extract had largely faded by the time this picture was taken (about 48 hours post mortem). (After Meites, J., and Turner, C. W. 1947. *Am. J. Physiol.* **150**, 394.)

described above, that luteotropin is *a* lactogenic hormone and plays an important role in lactation.

6. Progestational Effect

Early in the investigations of the physiological effects of adenohypophyseal extracts, evidence was obtained that these hormone-containing preparations were capable of suppressing the estrus cycle. It was concluded, therefore, that the factor, or factors, responsible were functioning by maintaining a secreting corpus luteum. Early fractionation work indicated that the hormone exhibiting this progestational effect was closely related to luteotropin, as judged from the properties, chiefly solubility, of the various fractions obtained from crude extracts (Dresel, 1935; Lahr and Riddle, 1936; Nathanson *et al.*, 1937; Lyons *et al.*, 1941). On the basis of their studies, Astwood and Fevold (1939), and Astwood (1941) were the first to suggest that while the luteinizing hormone (LH) is responsible for the conversion of follicles into corpora lutea, a third hypophyseal gonadotropic factor, distinct from luteinizing hormone, is involved in the maintenance of luteal function. Astwood (1941) suggested the name, "hypophyseal luteotrophin," for this hypophyseal principle, and succeeded in preparing a purified fraction from sheep hypophyses and in freeing it from follicle-stimulating, luteinizing, and thyrotropic hormones. In addition, evidence was presented that "hypophyseal luteotropin" was distinct from the growth and adrenotropic factors and, in Astwood's experiments, was not responsible for the proliferation of mammary lobules in hypophysectomized rats.

The chemical identity of the third gonadotropic hormone was established in Evans' laboratory in 1941. For several years, Evans and his associates had been studying the hypophyseal factors necessary for the production of functional corpora lutea in the rat, as judged by the ability to produce deciduomatous tissue in the traumatized endometrium. Of all the purified hypophyseal fractions investigated by the placentoma test, it was found (Evans *et al.*, 1938; Evans *et al.*, 1941a, b) that only their purified lactogenic (luteotropin) preparations would stimulate the production of progesterone by normally occurring or artificially induced luteal tissue in the rat. The progestational effects of luteotropin have been confirmed and extended by a variety of experimental procedures in numerous laboratories. Tobin (1942) observed that normal and adrenalectomized female rats showed maintenance of corpora lutea, which apparently secreted progesterone, when the animals were injected with luteotropic hormone. Further support for the identity of the hormone which exhibits both a lactogenic and a progestational effect has been presented by other investigators (Lyon, 1942; Everett, 1944; Sydnor, 1945; Desclin, 1946; Gaarenstroom and de Jonghs, 1946). Successful use of luteotropin has been reported in both menorrhagia (Hall, 1942; Kupperman *et al.*, 1944) and in alleviation of the signs and symptoms of threatened abortion (Kupperman *et al.*, 1944).

7. *Other Metabolic Effects*

A variety of metabolic effects have been described as a consequence of the administration of luteotropin. The major portion of these studies has been conducted in birds; surprisingly little investigation has been concerned with possible metabolic actions of luteotropin in mammals. This seems particularly unusual in view of the fact that a hormone which can induce and maintain lactation, and therefore affect the metabolic processes related to lactation (salt, water, lipide, protein, and carbohydrate metabolism) would be expected to affect the rates of a number of metabolic transformations.

a. Birds. The capacity of luteotropin to stimulate the crop-sac of pigeons has been discussed previously. In addition to this action, a variety of other effects of luteotropin have been obtained in pigeons. Among these are: a) the stimulation of broodiness and maternal behavior in hens of suitable genetic constitution and in active egg production, b) an antigonadal action, c) a marked calorigenic action, d) an increase in the body weight gain of either normal or hypophysectomized doves or pigeons, e) splanchnomegaly, characterized by a marked increase in the length and weight of the intestines of pigeons, f) an augmentation of the blood sugar level, and g) a marked effect on liver lipides. These various effects have been discussed in detail by Riddle and Bates (1939) and by Riddle (1947), who, with their associates, have contributed practically all of the available data bearing on these topics. The adrenal cortex apparently does not mediate either the growth-promoting effect (Miller and Riddle,1943) or the calorigenic action (Riddle and Smith, 1940) of luteotropin. The hormone has also been reported to stimulate the beta cells of the pancreas of pigeons (Miller, 1942).

b. Mammals. Luteotropin apparently also affects pancreatic cells of the mammal, although divergent results have been reported. In one laboratory (Fraenkel-Conrat *et al.*, 1942), luteotropin injections increased the insulin content of the pancreas in normal and in hypophysectomized rats. On the other hand, luteotropin has been reported (Funk *et al.*, 1941) to decrease the insulin content of the rat pancreas. This suggested influence on carbohydrate metabolism in the mammal is of interest in view of the recent observation by Reiss (1947) that luteotropin administration causes a reduction in the peripheral fat deposits of rats. This appeared to be a specific effect of this hormone, since thyroid treatment alone had no effect on fat deposits but, when given with luteotropin, could enhance the action of the latter.

VI. Concluding Remarks

The luteotropic hormone of the adenohypophysis has been isolated in highly purified form, and is a protein whose physical and chemical properties have been rather extensively characterized. The complete amino acid composition of luteotropin is now known, and the data demonstrate that no nonamino acid moiety is present in the hormone. A variety of agents which modify the structure of the protein, and cause either physical or chemical alterations in the molecule, also partially or completely destroy its biological activity. The conclusion is warranted, therefore, that the physiological behavior of the protein hormone is dependent on its properties as a protein, per se, and cannot be attributed to a particular or special portion of the molecule.

In addition to its classical ability to initiate lactation, luteotropin has the capacity to stimulate proliferation of mammary gland tissue. This hormone must also be classed as one of three hypophyseal gonadotropins, inasmuch as it has a distinct progestational action; in the absence of luteotropin, there is a failure of continued functioning of the corpus luteum.

Evidence has accumulated, for the most part in birds, and has yet to be obtained in mammals, that luteotropin may exert a diversity of metabolic effects. The indicated physiological actions of the hormone in birds, as well as in mammals, make it likely that hypophyseal luteotropin must have a profound influence, either direct or indirect, upon many processes of cellular metabolism. With the availability of highly purified preparations of the hormone, one may expect a stimulation of interest in its possible physiological actions, other than those attending lactation, as well as a re-evaluation of those metabolic alterations which have been described to result from the administration of less purified luteotropin products.

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Infrared Spectrometry Applied to Steroid Structure and Metabolism

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I. INTRODUCTION

The steroid hormones play an integral part in the control of the development and activity of the animal organism. They regulate the sex function through the action of the male sex hormone (testosterone), the estrogenic hormones (estrone, estradiol), and the corpus luteum hormone (progesterone). In addition a large number of steroids have been isolated from the cortex of the adrenal gland (Reichstein and Shoppee, 1943) and these are essential for the maintenance of life. Both the sex hormones and the adrenocortical hormones are involved in the regulation of protein, carbohydrate, and electrolyte metabolism.

In an endeavor to gain information about their precursors and the regulatory mechanisms which control their action, an increasing number of investigators are studying the metabolites of these hormones in the excreta (Pincus and Pearlman 1943). The steroids excreted in the urine of human beings in varying physiological and pathological states have been the subject of investigation at the Sloan-Kettering Institute of The Memorial Hospital, New York, and methods have been developed for the separation, isolation and identification of the urinary steroids (Dobriner *et al.*, 1948b; Lieberman *et al.*, 1948a). In the course of this work extremely complex mixtures were encountered, and it was evident that the conventional methods of chemical analysis were inadequate for the identification of *all* the steroid metabolites, many of which occur in exceedingly small amounts. A more sensitive method of analysis was therefore sought; in 1943 Dr. R. Bowling Barnes and Dr. V. Z. Williams of the American Cyanamid Company suggested the use of infrared spectrometry, and this, when employed in conjunction with adsorption chromatography, effectively overcame the difficulties involved in the fractionation of these complex mixtures and the identification of their constituents.

An analytical technique has been developed (Dobriner *et al.*, 1948b, 1948d) in which the mixtures are partially or completely separated chromatographically; the separations are monitored by infrared spectrometry so that a complete knowledge of the progress of the fractionation is available at every stage. Infrared spectrometry is now employed extensively, not only for the isolation of steroids from natural sources, but also in connection with problems of steroid synthesis and structure, and in the study of the physical and chemical properties of the individual steroids (Jones *et al.*, 1948a, 1948c, 1949).

The object of this article is to illustrate how infrared spectrometry has been applied to the following problems:

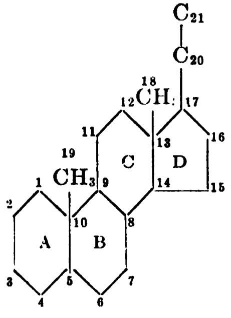
(a) The identification of individual steroids by empirical comparison with the spectra of known steroids.
(b) The separation of mixtures of steroids and the recognition of new steroids in such mixtures.
(c) The quantitative analysis of steroids and steroid mixtures.
(d) The detection and assay of steroids labeled with deuterium.
(e) The elucidation of the structure of steroids.

II. THE CHEMISTRY OF THE STEROIDS

The chemistry of the steroids constitutes a voluminous and highly specialized division of organic chemistry; it is not proposed to discuss this in detail here, or to present the evidence on which the accepted formulations of the steroid hormones are based. For this, reference should be made to the monographs by Fieser and Fieser (1949) and Strain (1942), and to special articles by Mason (1948), Reichstein and Shoppee (1943), and Shoppee (1946, 1947). Certain features of steroid chemistry are of special importance in connection with the hormones and hormone metabolites, however, and some discussion of these is necessary.

1. The Carbon Skeleton

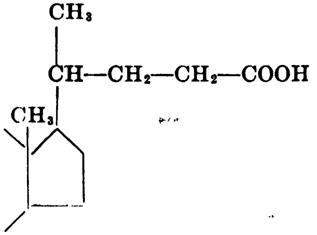
The steroid molecule consists of a framework of three six-membered and one five-membered rings (I), with methyl groups at positions 10 and 13. In the estrogens ring A is aromatic and the methyl group at 10 is lacking.



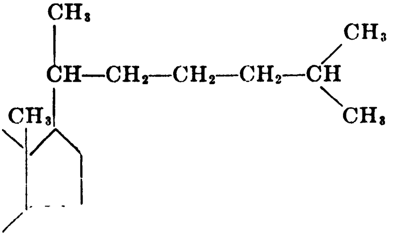
I

The compounds in which no side chain is present on carbon atom 17 constitute the "C_{19} series." This includes the male sex hormone, testosterone, and its urinary metabolites. Steroids with a side chain of two carbon atoms, the "C_{21} series," include the progestational hormone (progesterone), and the hormones of the adrenal cortex.

In the C_{24} series a branched side chain (II) is present; this is the characterizing feature of the bile acids. Compounds in the C_{27} series contain a side chain of eight carbon atoms, typified by that of cholesterol and the precursors of vitamin D_3 (III).



II

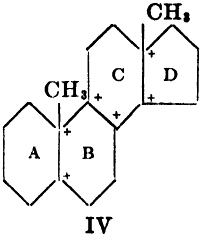


III

2. *Ring Isomerism and Stereochemistry*

The carbon atoms at each of the ring junctions in the steroid nucleus normally carry an additional substituent; methyl groups are at positions 10 and 13, and hydrogen atoms at positions 5, 8, 9 and 14. The geometry of the C—C bond joining the two rings permits two spacial arrangements of these substituents, one in which both the substituents are located on the same side of the plane of the ring system (*cis*-configuration) and a second in which the two substituents are on opposite sides of the plane of the ring (*trans*-configuration).

Altogether there are six centers of stereoisomerism in the steroid ring system, indicated by crosses in IV. These could theoretically give rise to 64 stereoisomers, but the number of ring stereoisomers actually encountered in naturally occurring compounds is considerably less. The steric arrangements at the B/C and C/D ring junctions are identical in all but a very few of the naturally occurring steroids.*



IV

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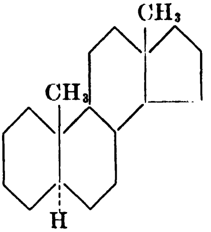
* In the uranes, Marker *et al.* (1938) have suggested that the B/C ring junction differs from that normally encountered. The stereoisomerism of the C/D ring junc-

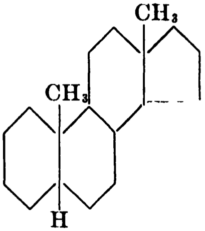
The two centers of isomerism at 5 and 10 carry a hydrogen atom and a methyl group respectively and may give rise to four stereoisomers. These constitute two pairs, one pair with *cis*-configuration and one pair with *trans*, as shown in V–VIII. Since the *absolute* configuration of no steroid is yet known, the naturally occurring isomers are conventionally represented as having the methyl group at 10 projecting *above* the plane of the ring system, indicated by a solid line; all other stereochemical relationships are referred to this.

The naturally occurring steroids in the C_{19} series may accordingly be regarded as derivatives of the two basic hydrocarbons, androstane (V) in which the A/B ring junction substituents are *trans*, and etiocholane (VI) in which these substituents are *cis*. Formulas VII and VIII represent stereoisomeric forms which do not occur in natural products and have not yet been synthesized.



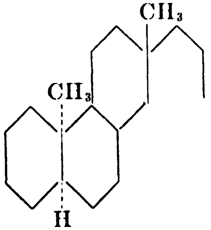
Androstane
(*Trans*)

V



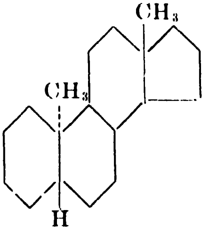
Etiocholane
(*Cis*)

VI



(*Cis*)

VII



(*Trans*)

VIII

The naturally occurring steroids in the C_{21} series may likewise be considered as derivatives of the basic hydrocarbons allopregnane (IX) with a *trans*-configuration of the A/B ring junction substituents, and

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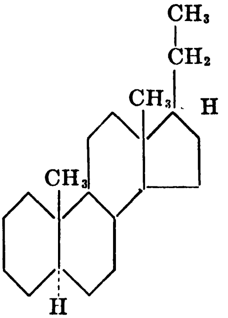
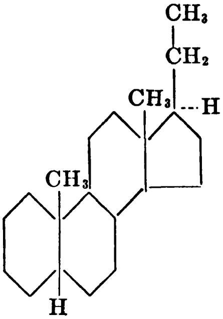
tion has been extensively investigated in connection with the preparation of synthetic equilenin and estrone derivatives, and of products related to doisynolic acid. These studies have been reviewed recently by Shoppee (1946, 1947) pregnane (X) with the *cis*-configuration. In this case, however, additional isomerism is introduced by the side chain at 17 which is discussed below.

In view of the fact that numerous interconversions of naturally occurring steroids in the C_{19} and C_{21} series have been carried out, it is established that the absolute configurations at the A/B ring junction (and elsewhere) are the same in both series.



Allopregnane

IX



Pregnane

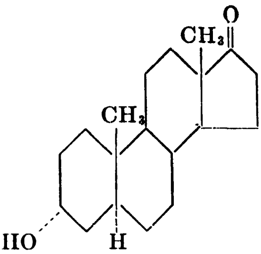
X

3. *Stereoisomerism Associated with Substituents and the Side Chain*

In the —CH_2— groups of the ring system, the two hydrogen atoms project one above and one below the plane of the molecule. If one of these hydrogens be replaced by some other group, a new center of asymmetry is introduced. Two structures are possible for the derivative, one in which the group projects below the plane of the ring (XI) (dotted line) and one in which it projects above (XII) (solid line).

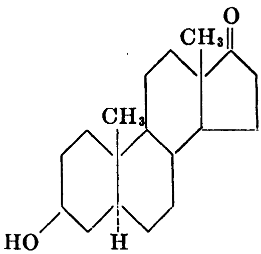
Many steroids carry a hydroxyl group at position 3. In order to designate the two series of 3-substituted stereoisomers, Fieser introduced the use of the symbols α and β to indicate the stereochemical relationship of the hydroxyl group at 3 to the rest of the molecule. The configuration β was originally defined as that present in cholesterol, and the opposite α-configuration that present in the bile acids and in androsterone. In the β-compounds the hydroxyl group lies on the same side of the ring as the methyl group at 10, and accordingly it is indicated by a solid line. This has been proved recently by Shoppee (1947).

In the naturally occurring C_{19} series this gives rise to four derivatives, two from androstane and two from etiocholane. The 17-ketosteroids containing all four configurations occur in urine and are shown in XI–XIV.



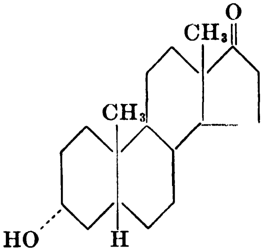
Androstanol-3α-one-17
(Androsterone)

XI



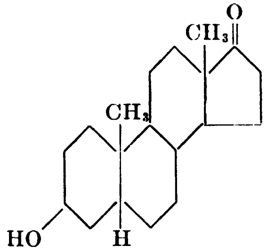
Androstanol-3β-one-17
(Isoandrosterone)

XII



Etiocholanol-3 α-one-17
(α-Etiocholanolone)

XIII



Etiocholanol-3 β-one-17
(β-Etiocholanolone)

XIV

Substituents in the C and D rings are related similarly to the configuration of the methyl group at 13.* Thus the naturally occurring 11-hydroxy compounds in which the hydroxyl group is *cis* to the methyl group at 13 are designated 11β-hydroxy compounds (Long and Gallagher, 1946).

In the C_{21} series, steroisomerism of the same type may arise at position 17 where the two-carbon-atom side chain may project above or below the plane of the ring. The absolute configuration of the side chain in the pregnane and allopregnane compounds has been a subject of controversy, but recent work of Sorkin and Reichstein (1946) and of Long and Gallagher (1946) indicates that the naturally occurring C_{21}

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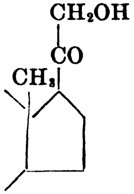
* By convention, the angular methyl group (C_{19}) attached to C_{10} is β-orientated; it can be shown that the 12 hydroxyl group in desoxycholic acid (Sorkin and Reichstein, 1946; Long and Gallagher, 1946) is α-orientated, and, since this hydroxyl group is known to be *trans* to the angular methyl group (C_{18}) attached to C_{13}, it follows that *both* angular methyl groups are β-orientated. It may be noted also that any other arrangement raises difficulty from the crystallographic standpoint in relation to the packing of steroid molecules into unit cells.

steroids have a β-configuration, with the side chain *cis* to the methyl group at 13 (IX, X).*

4. *Substituents Commonly Occurring in Steroid Hormones and Metabolites*

a. Hydroxyl Groups. Hydroxyl groups are present in many of the steroid hormones and metabolites isolated. In addition to the 3 position, hydroxyl groups are frequently observed also at positions 17 and 20. Many of the steroids derived from the adrenal cortex also carry a hydroxyl group at position 11 as well as a hydroxyl group at 21 as part of the characteristic ketol side chain (XV). In the bile acids hydroxyl groups at 6, 7 and 12 occur.



XV

b. Carbonyl Groups. The *ketone* group occurs in many steroids, usually at the same positions as the hydroxyl group, i.e., 3, 7, 11, 12, 17, and 20. There is considerable evidence to show that, in the course of metabolism, oxidation and reduction between the hydroxyl groups and ketone groups at various positions in the molecule may take place (Dobriner and Lieberman, 1949a), (Lieberman and Dobriner, 1949a). In the naturally occurring steroids the *carboxylic acid* group is present in the bile acid side chain (II).

c. Unsaturation. An additional factor which contributes to the complexity of the natural occurring steroids is the frequent presence of ethylenic unsaturation in the molecule. *Isolated double bonds* can occur at a variety of positions. They are found frequently at the Δ^5-position,† as in cholesterol and dehydroisoandrosterone, and have been observed also at the Δ^2 (or Δ^3), $\Delta^{9:11}$, Δ^{11}, Δ^{16} positions, and on the side chain.

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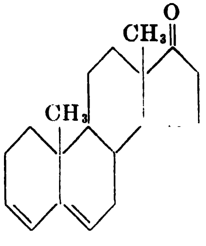
* Complete agreement concerning steroid nomenclature has not yet been achieved and the various prefixes such as "iso," "epi," etc., are often used by different investigators in a slightly different sense (see Fieser and Fieser (1949), Heard and Prins (1948), Mason (1947), Selye (1943)). The nomenclature used in this article conforms with that employed in our earlier publications.

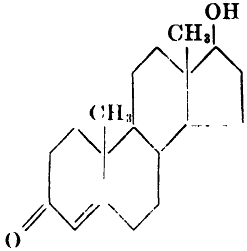
† When the symbol Δ is used to indicate the position of a double bond in the molecule, only one numerical superscript is shown if the opposite end of the double bond is attached to the carbon atom carrying the consecutive higher number; otherwise both ends of the bond are indicated, the numbers being separated by a colon. In the case of a diene, a comma is used to separate the numbers locating each of the two bonds.

Conjugated Ethylenic double bonds are found also in 7-dehydrocholesterol, the precursor of vitamin D_3, and in the urinary metabolite $\Delta^{3,5}$-androstadienone-17 (XVI). Conjugation of an ethylenic double bond with a ketone group very frequently occurs, particularly in the Δ^4-3 ketone arrangement (XVII) which is found in progesterone, testosterone and the active steroids of the adrenal cortex. The presence of an *aromatic ring* A is characteristic of the estrogens. This confers phenolic properties on the hydroxyl group at 3.



($\Delta^{3,5}$-Androstadienone-17)

XVI



(Testosterone)

XVII

III. The Principles of Infrared Spectrometry

1. The Nature of Infrared Absorption Spectra

For most purposes of organic and biochemistry, a covalently bonded molecule can be considered as a rigid structure each atom of which occupies a fixed position with respect to the other atoms of the molecule, and is attached to them by bonds of fixed length oriented at fixed angles. It is on such a concept that the conventional structural formulas of organic chemistry are based. This represents a considerable oversimplication of the true state of the molecule, as even in the lowest energy state the constituent atoms of the molecule are in constant vibratory motion about their mean positions. In a more exact representation the rigid bonds of the conventional structural formula would be replaced by springs, the tensions of which would differ in the various types of interatomic bonds.

When the molecule is exposed to infrared radiation, energy is absorbed and the molecular vibrations excited. Such absorption will occur only if the frequency of the radiation is suitably related to the frequency of vibration of the atoms of the molecule. If the radiation is continuous certain frequencies only will be absorbed and an absorption spectrum will result.

The complete infrared region of the spectrum extends from the red end of the visible spectrum to the beginning of the microwave region.

The vibration spectra which are considered here extend from about 1 μ to 100 μ although because of instrumental limitations only the region from 2 μ to 25 μ is normally investigated. In Fig. 1 these various regions of the spectrum are illustrated diagrammatically.

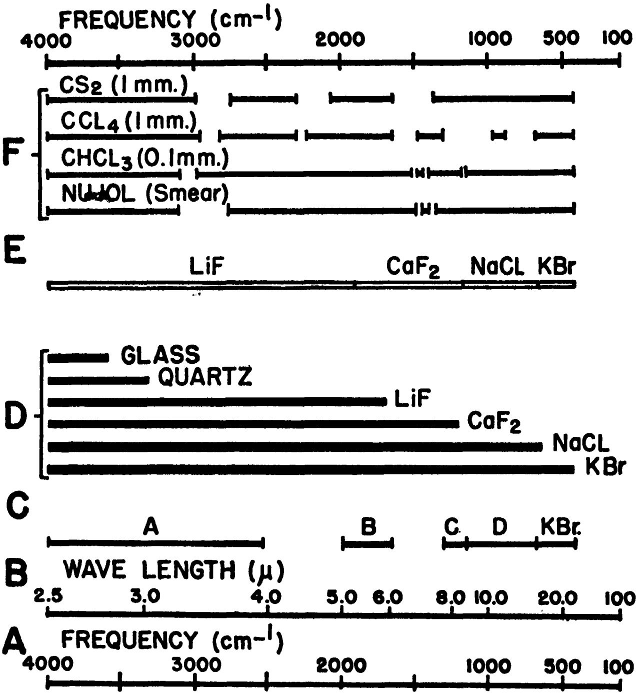


FIG. 1. Diagram illustrating transmission characteristics of optical materials and solvents in the infrared region of the spectrum.

A. Frequencies in wave numbers.

B. Wavelengths in microns.

C. Regions of the spectrum arbitrarily defined for the description of steroid spectra.

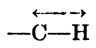
D. Limits of transparency of optical materials.

E. Prism materials for optimum dispersion. (The NaCl prism may be used over the whole region from 4000 to 650 cm.$^{-1}$, but prisms of LiF and CaF_2 have considerably greater dispersion in the regions indicated; the dispersion of KBr is poor at frequencies greater than 1000 cm.$^{-1}$)

F. Commonly employed solvents; regions of transparency are indicated by solid black line.

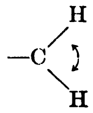
In a large molecule, such as a steroid, very complex interactions can occur between the vibrational motions of one atom and those of its neighbors. In consequence of these interactions, new oscillations arise which are associated with groups of atoms, or with the molecule as a whole. These vibrations also will give rise to absorption bands in the spectrum, so that it is not necessarily to be expected that all the various absorption bands observed in the spectrum can be simply related to specific bonds or atoms in the molecule. Any change in the masses of the atoms or in their relative positions in the molecule will disturb the delicately adjusted vibrational system and hence alter the infrared absorption spectrum. Because of this sensitivity to small structural changes, the infrared spectrum is probably the most specific of all the physical properties of a molecule (Sutherland and Thompson, 1945).

This specificity does not extend to all of the absorption bands. The vibratory motions of the atoms can be classified into two general types: a longitudinal stretching motion of the bond (XVIII), and a transverse bending motion of the bond (XIX). In most cases it requires more energy to change the length of a bond than to deflect the bond angle, and absorption bands associated with stretching motions tend to occur at the higher frequencies, while the bending motions occur at the lower frequencies.



$\overset{\longleftrightarrow}{—C—H}$

XVIII



XIX

Certain of the stretching motions do not interact to any considerable extent with the other vibrations of the molecule and can be clearly recognized at the higher frequency end of the spectrum. This is true particularly of the stretching vibrations of the O—H, C—H, C—D, C=O and C=C bonds. The recognition of these structural groupings by their characteristic infrared absorption bands has been utilized extensively in the elucidation of the structure of steroid molecules and is discussed in detail in Section V.

2. *The Measurement of Infrared Absorption Spectra*

Spectrometers used for the measurement of infrared absorption spectra do not differ in fundamental principles from those employed in visible and ultraviolet spectrometry, but certain physical limitations of the commonly used optical materials necessitate some radical differences in design.

It is seen from Fig. 1 that the transmission limits of glass and quartz occur at the very beginning of the useful region of the infrared and it is necessary to resort to prisms and windows made of halide salts. These are prepared from large single crystals grown synthetically. For most purposes sodium chloride is employed. Potassium bromide permits extension of the measurable range further to lower frequencies, while calcium fluoride and lithium fluoride are used as prism material at the higher frequencies when the maximum resolution is desired. Higher dispersion may be obtained by the use of gratings, but prismatic dispersion is utilized in the commercial instruments currently available.

The halide salts do not lend themselves to the construction of achromatic lenses, and in infrared spectrometers mirrors are invariably used for the focusing and collimation of the radiation. In view of the hygroscopic nature of sodium chloride and potassium bromide, special precautions have to be taken to protect infrared spectrometers from conditions of high humidity and they are preferably operated in an air-conditioned laboratory in which the humidity can be maintained below 50%.

The radiation is provided by a heated rod of silicon-carbide (a "globar") or a Nernst filament, both of which emit essentially black body radiation at about 1500°C. Thermocouples, or, less commonly, bolometers are used to measure the transmitted radiation. The thermocouple currents are amplified electronically and recorded on a strip-chart potentiometer. In the most recent instruments the radiation is interrupted by a rotating sector to give a low frequency alternating signal instead of a direct current signal. This has an advantage of giving greater stability in the amplifier and yields a spectrographic record not subject to "drift" which otherwise is incurred as a consequence of ambient temperature changes in the instrument.

Several infrared spectrometers are now available commercially. In the work carried out in our laboratories both in New York and Ottawa, Perkin-Elmer instruments have been employed (Barnes *et al.*, 1945). Several review articles dealing with various phases of infrared instrumentation have appeared in the literature and reference should be made to these for more detailed information (Barnes *et al.*, 1944; Sutherland and Thompson, 1945; Williams, 1948).

In applying infrared spectrometry certain limitations must be kept in mind. The absorption spectrum is apt to be influenced by the physical state of the substance, and spectra in the solid and liquid states and in solution cannot be directly compared with one another. For most purposes it is preferable to work in solution, particularly in quantitative studies where a knowledge of the concentration and sample thickness is needed. Difficulty is encountered because of the very limited number of suitable solvents (Torkington and Thompson, 1945). Indeed, for the survey of extensive regions of the spectrum carbon disulfide is the only suitable solvent.* If the substance is insoluble in carbon disulfide it is necessary either to resort to the use of a crystalline film deposited on a sodium chloride plate, or prepare a mull by grinding the material in the presence of liquid petroleum and smearing the resultant mull between two sodium chloride plates.† Another alternative is to convert the substance to a derivative soluble in carbon disulfide. This latter technique has been adopted frequently; hydroxy steroids such as the pregnanediols, androstanediols and estrogens which are insoluble in carbon disulfide may be examined as the soluble diacetates. The estrogens have also been investigated by Carol *et al.* (1948) as the benzenesulfonyl esters.

If only narrow regions of the spectrum are to be investigated other solvents may be employed. Carbon tetrachloride and chloroform may be used over extensive regions at the higher frequency end of the spectrum. Chloroform in thin layers (0.1 mm.) may also be utilized at lower frequencies. A variety of other solvents, suitable for use in selected narrow regions of the spectrum have been listed by Torkington and Thompson (1945).

The small amount of material available for analysis is a factor of considerable importance in many biochemical studies. This difficulty may arise not only in connection with material isolated from natural sources but also in regard to material produced synthetically. By the use of suitably designed micro-cells (Colthup and Williams, 1947; Hardy, 1949) it is possible to determine the infrared spectrum on 25 γ or less of material in solution, whereas to prepare a suitable crystalline film or Nujol mull, at least 2 mg. are required. In the case of the mull, recovery of the material by separation from the added Nujol is impracticable in most cases.

IV. The Infrared Spectra of Steroids

1. *Principal Features of Steroid Spectra*

The infrared absorption spectrum of cholesterol was recorded by Lecomte (1943) and infrared spectrometry has been employed by Stora and Freymann (1939) in an investigation of the molecular association of cholesterol in different solvents. Recently Furchgott *et al.* (1946a, b, c,

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* The intense absorption of water vapor over considerable regions of the spectrum makes the analysis of hydrated and deliquescent substances particularly difficult, and in some instances impossible. Such conditions were not encountered in the investigation of the substances discussed in this article.

† The commercial product "Nujol" is very suitable for this purpose and these preparations are commonly referred to as "Nujol mulls."

1947a, b) have described the spectra of solid films of a variety of steroid hormones and metabolites.

In Fig. 2 is shown the spectrum of androsterone (XI) determined in carbon disulfide solution using a sodium chloride prism. The intensities are expressed as "per cent absorption" and the band positions in wave numbers.

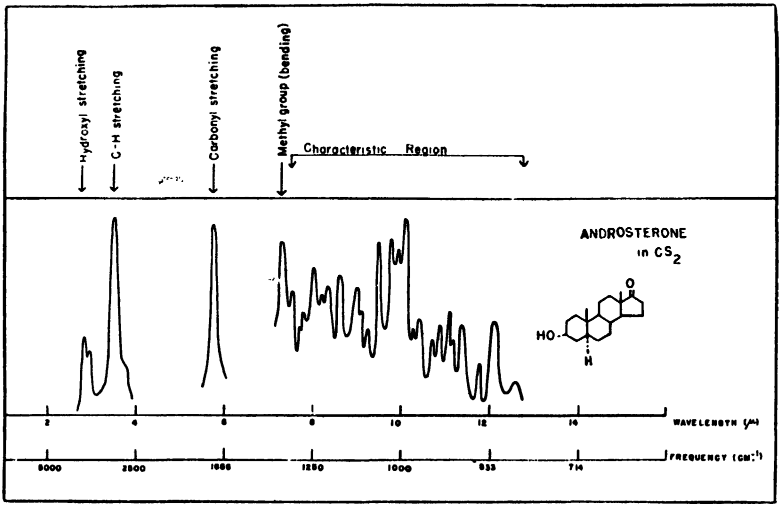


Fig. 2. Infrared spectrum of androsterone in carbon disulfide solution.

Up to the present time, no standard system has been agreed upon for the recording of infrared data. Intensities are reported by various investigators as *per cent absorption* $\frac{(100(I_0 - I))}{I_0}$, *per cent transmission* $\left(100\frac{I}{I_0}\right)$, or "*optical density*" $\left(\log_{10}\cdot\frac{I}{I}\right)$, where I_0 is the intensity of the incident radiation and I the intensity of the transmitted radiation. For most purposes we employ percentage absorption but use optical densities in problems involving the evaluation of absolute absorption intensities (page 330) and in quantitative analysis (page 355). If the per cent transmission scale is used, the curves are inverted so that the absorption maxima appear as "transmission minima."

With regard to the designation of band positions, usage is fairly evenly divided between expression on a wavelength or on a frequency scale. On the wavelength scale the unit customarily employed is the micron (μ). Frequencies are expressed in wave numbers (cm.$^{-1}$). The wave number is the number of waves per centimeter *in vacuo*. The wave number and wavelength are related by the equation:

$$\nu = \frac{1}{\lambda} \times 10^4$$

where ν is the frequency in wave numbers and λ the wavelength in microns.

For the purposes of classification and discussion, it has been found convenient to divide the steroid spectra into four regions designated A, B, C and D in Fig. 1. The absorption maxima in the A, B and C regions can be associated with specific chemical groupings in the molecule

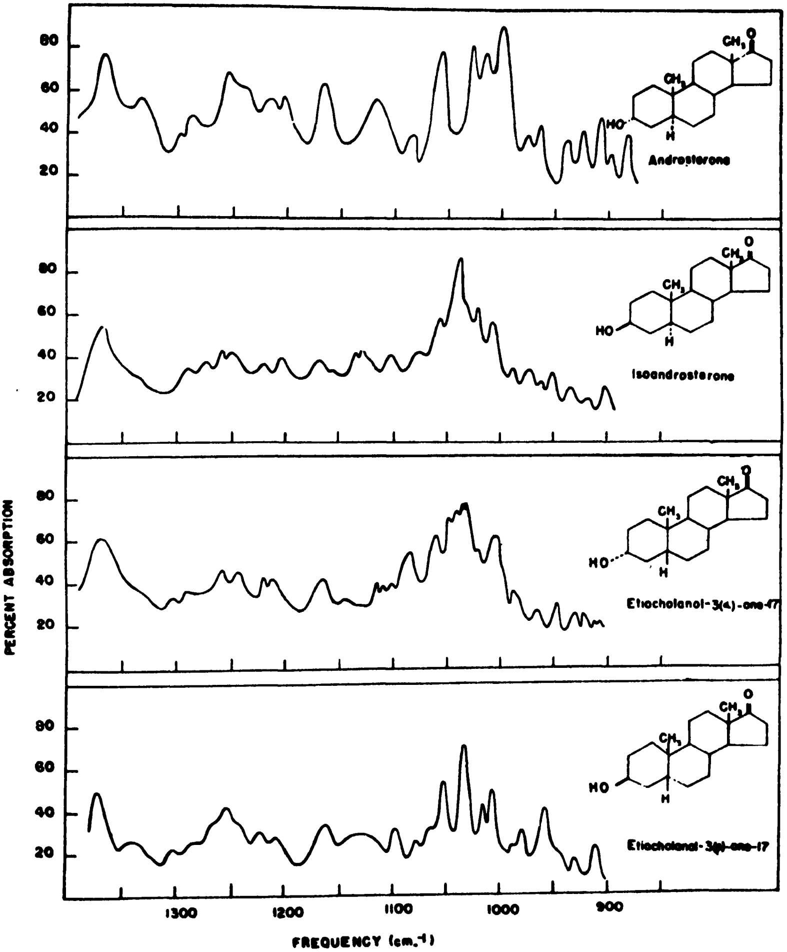


FIG. 3. Infrared spectra of the four naturally occurring stereoisomeric 3-hydroxy-17-ketosteroids in the "fingerprint" region.

as indicated. In the D region of the spectrum a multitude of overlapping absorption bands occur. The bands in this region are associated with vibrations greatly subject to effects of interaction so that any change in the molecular structures causes modifications in the appearance of these absorption bands. This part of the spectrum has accordingly been

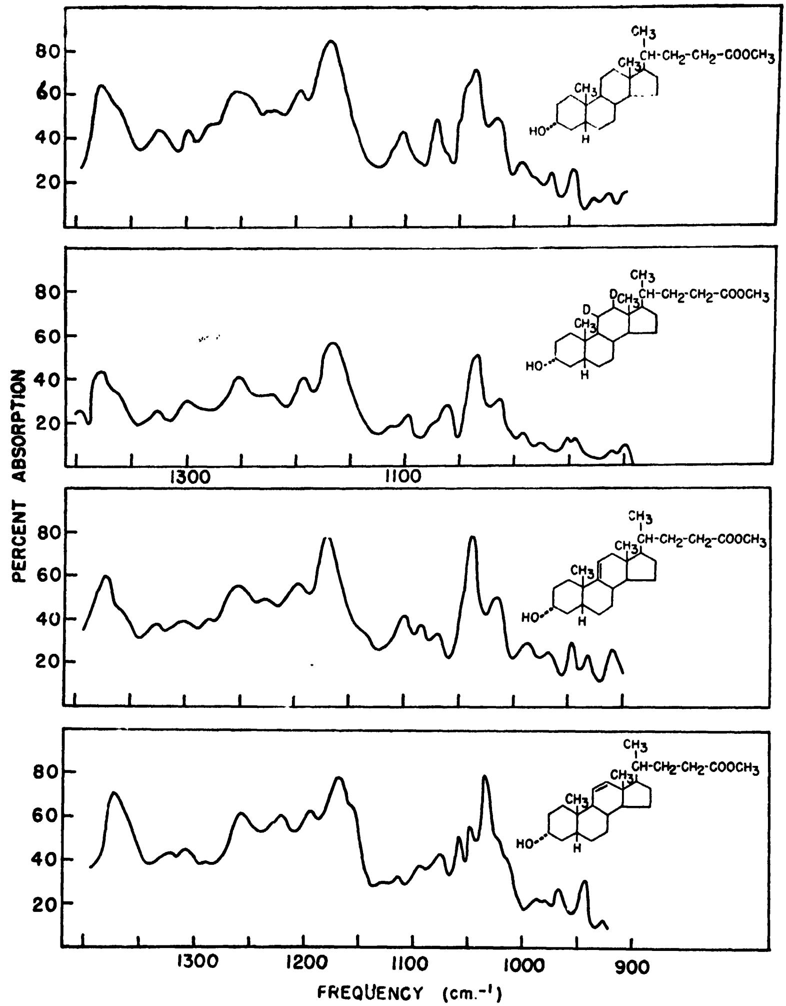
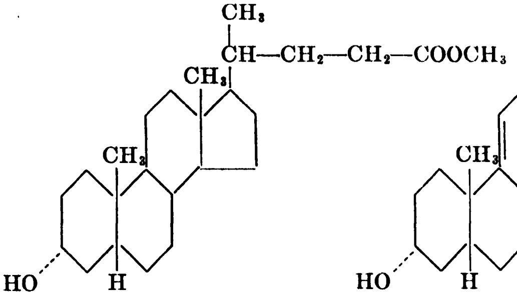


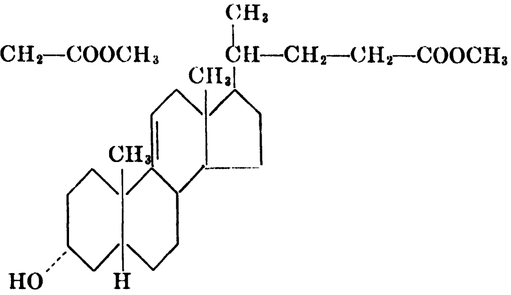
Fig. 4. Spectra of cholanic acid methyl ester derivatives illustrating the effect of the introduction of unsaturated linkages and of deuterium in ring C.

called the "fingerprint" region (Barnes *et al.*, 1944). Insofar as the steroids are concerned this name is fully warranted, as a comparison of the curves of over 500 steroids has failed to show identity in any two of them.

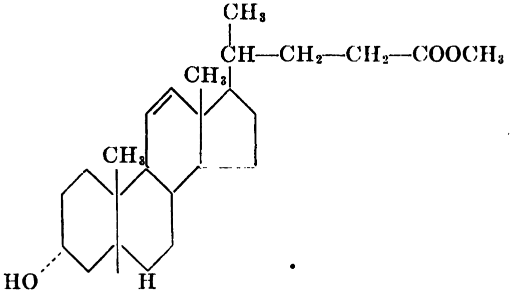
An illustration of the specificity of the absorption in this fingerprint region is provided by the four curves in Fig. 3. The spectrum of androsterone is compared with those of three stereoisomers (XI–XIV) which differ from it only in the steric configurations at positions 3 and 5. Minor differences in chemical structure lead to comparable changes in the absorption spectrum, as is illustrated in Fig. 4 where the spectra of the three steroids XX, XXI and XXII are shown. Compound XX is saturated, while XXI and XXII differ by the presence of a $\Delta^{9:11}$ or a $\Delta^{11:12}$ double bond. Whereas the major bands in these spectra are at approximately the same positions, and of comparable intensity, considerable differences are to be seen in the number and location of the weaker bands present in the spectra of these three compounds. It follows from this that the ability to make such differentiations is dependent in many instances on having sufficient resolving power in the spectrometer.



XX



XXI

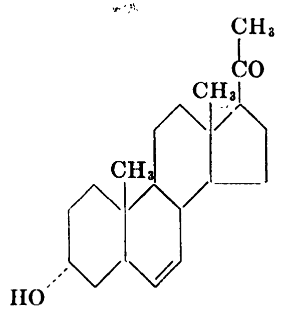


XXII

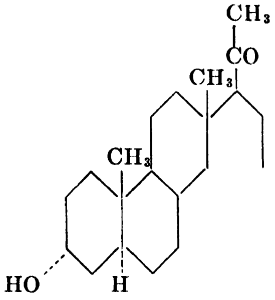
It does not necessarily follow that with a continued increase in the resolving power, additional structure would continue to appear in the spectrum. These absorption bands possess a certain finite width and once the resolution of the spectrometer exceeds a limiting value, no additional structure will be revealed. In simple molecules at high resolution, the vibrational absorption bands often split up to reveal rotational fine structure. However such rational bands do not occur in the spectra of large molecules in solution. Some further gain in resolution can be achieved by measuring the spectrum at liquid air or liquid helium temperatures but the technical difficulties involved are considerable.

Any change in the physical state of the molecule will influence the absorption curve in this finger print region, to a greater or less extent. Accordingly in comparing such spectra it is important that the compounds be examined in the same physical state, and, where conditions permit, it is preferable to record the spectra in dilute solution (1–2%) in carbon disulfide.

An example of the change in the spectrum of the solid with change in the crystalline form has been reported by Haines *et al.* (1948) for Δ^5-pregnenol-3-α-one-20 (XXIII) and confirmed in our laboratories. Similar behavior has also been observed for two polymorphic forms of allopregnanol-3-α-one-20 (XXIV).



XXIII



XXIV

2. *The Introduction of Isotopes*

The effect of isotopic change on the infrared spectrum is of both theoretical and practical interest. The frequencies of the molecular vibrations responsible for the appearance of infrared absorption bands (see Section III) are dependent on the masses of the atoms involved. Any change in the atomic mass will change the frequency of vibration; an increase in mass will slow the vibration and displace the absorption band to a lower frequency.

The magnitude of the displacement depends on the change in the ratio of the masses of atoms involved. In the case of the stretching vibration bands, the approximate frequency of the vibration (ν) is given by the equation

$$\nu = \frac{1}{2\pi}\sqrt{\frac{f}{\mu}}$$

where f is the force constant of the bond, and

$$\frac{1}{\mu} = \frac{1}{m_1} + \frac{1}{m_2}$$

where m_1 and m_2 are the masses of the atoms involved.

In the C—H and O—H stretching vibrations, replacement of hydrogen by deuterium results in an increase of m_1 from 1 to 2. From the equations given above, it follows that the band maximum will be displaced from ν_1 to ν_2 where

$$\nu_2 = \sqrt{2}\,\nu_1$$

By application of the same equation it can be readily shown that replacement of C^{12} by C^{13} will displace the C—H stretching band maxima by $\sqrt{\frac{169}{168}}$ and similarly, replacement of O^{16} by O^{18} will displace the O—H stretching vibration maximum by $\sqrt{\frac{306}{304}}$.

Although the effects on the infrared spectrum of the introduction of carbon and nitrogen isotopes into certain organic compounds have been detected (Sutherland, unpublished observation), it is only on the introduction of deuterium that large changes in the infrared spectra occur (Hardy *et al.*, 1932; Herget and Hardy, 1938). The replacement of a C—H bond by a C—D bond produces a primary effect on the C—H stretching vibration, and displaces the corresponding absorption band from 2800–3000 cm.$^{-1}$ to 2100–2200 cm.$^{-1}$ It also has a primary effect on the C—H bending vibrations, which occur in the region 725–1450 cm.$^{-1}$ and contribute to the complex absorption of the fingerprint region.

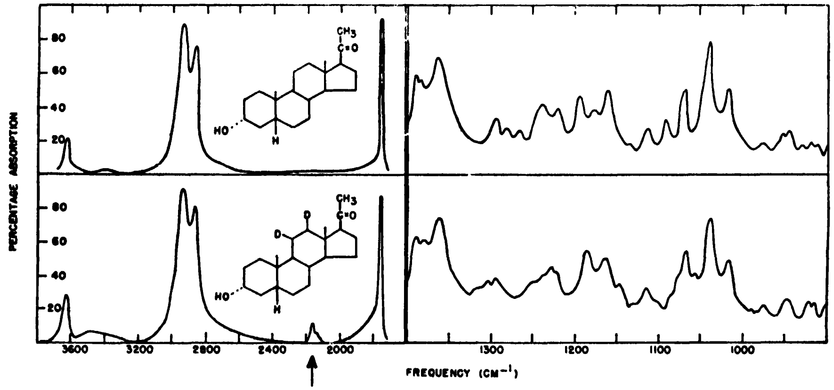


Fig. 5. Effect of introducing two deuterium atoms on the spectrum of pregnanol-3α-one-20.

In Fig. 5 the absorption spectrum of pregnanol-3-α-one-20 (XXV) is compared with that of the same compound with deuterium at positions 11 and 12 (XXVI) (Dobriner *et al.*, 1949b). The appearance of two new bands at 2165 and 2145 cm.$^{-1}$ is due to the C—D stretching vibration. Changes in the finger print region are also clearly to be seen.* The finger print region of a second steroid with deuterium atoms at 11 and 12 is included in Fig. 4.

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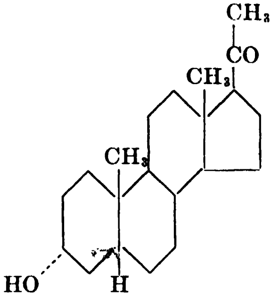
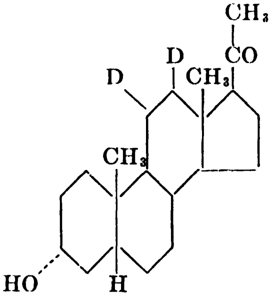
* In order to observe the C—D stretching bands a solution in chloroform or carbon tetrachloride must be used, as carbon disulfide absorbs strongly in this region.



XXV



XXVI

V. Correlation of Absorption Spectra and Steroid Structure

In a steroid one or more hydroxyl and carbonyl groups, and one or more ethylenic double bonds may be present. These characteristic groupings may be located at several different positions in the ring system or on the side chain. In addition to the extensive possibilities of structural isomerism which arise in this manner, the variety of possible structures is multiplied severalfold by the stereoisomerism of the ring system (see page 296).

In the preceding section it has been shown that in the region between 700 and 1200 cm.$^{-1}$ the complex infrared spectrum is highly specific and permits each steroid to be identified uniquely. With very few exceptions, however, it has not yet proved possible to relate the specific absorption bands in this region of the spectrum with individual features of the molecular structure. By and large, it would appear that these absorption bands are associated with molecular vibrations which cannot be assigned to isolated parts of the molecule. These oscillations result from a delicately balanced system of vibrating bonds, which interact on one another to such an extent that any change in one of the contributing vibrations disturbs appreciably the oscillation of the whole system.

It has been pointed out previously (page 303) that certain of the individual absorption bands, particularly those occurring at the higher frequencies, can be related to stretching vibrations localized in specific bonds (Barnes *et al.*, 1948; Thompson, 1948; Williams, 1948). Of particular importance in connection with the steroids are the bands associated with the O—H, C—H, C—D, C=O and C=C groups (Furchgott *et al.*, 1946a). By a comparative study of the positions and absolute intensities of these bands in a variety of steroids much information has been derived which has proved of distinct value in the elucidation of the structure of newly isolated or synthesized steroids.

1. The Carbonyl Stretching Vibration

Up to the present time the investigation of the carbonyl stretching vibration has yielded the most important and extensive information. Previous investigators have established from studies on a wide variety of organic compounds (Barnes *et al.*, 1944; Lecomte, 1925) that a characteristic intense absorption band between 1550 and 2150 cm.$^{-1}$ is given by all compounds which contain the C=O linkage. This is true whether the carbonyl group occurs as part of a ketone, aldehyde, carboxylic acid, carboxylic ester, lactone, acid anhydride, or amide group.

In the steroids, three types of carbonyl functions are commonly encountered; the ketonic carbonyl, the carbonyl of the carboxylic acid group, exemplified by the bile acids, and the carbonyl of an ester group such as the acetates, benzoates and other carboxy esters of steroid alcohols.

a. Monoketones. The ketonic carbonyl group occurs frequently at positions 3, 7, 11, 12, 17, and 20 in steroids, and in many instances two or more ketonic groups may be present in the same molecule; such ketone groups may or may not be conjugated with an ethylenic double bond.

A comparison of a large number of monoketosteroids (Jones *et al.*, 1948a, 1948c, 1949) has shown that the frequency of the absorption maximum of the C=O stretching vibration in these compounds is specific for a given position of the carbonyl group and if the carbonyl group is conjugated with an ethylenic bond, the location of the absorption maximum is displaced to lower frequencies.

In the following paragraphs, the relationships which have been established between the position of the carbonyl bands and the molecular structure are discussed in detail. A complete list of the observed correlations is given in Chart I.

Considering first the *nonconjugated* monoketones it will be seen that, in the five-membered ring, the carbonyl maximum occurs at a high frequency (1749–1742 cm.$^{-1}$) (Chart I structures ix, x). In the hexacyclic A, B and C rings the nonconjugated ketone group gives maxima lying between 1720 and 1710 cm.$^{-1}$ (xix, xx, xxii, xxiii), while in the open chain 20-ketone (xxiv) the maximum is at 1710–1706 cm.$^{-1}$ The small frequency difference between the maxima of 3, 7, 11, 12 and 20 nonconjugated ketones leads to some ambiguity in the differentiation between these groups; this is discussed further in connection with the spectra of diketones on page 326.

If the ketone group is *conjugated* with an ethylenic double bond, the

CHART I

STRUCTURAL GROUPS CHARACTERIZED BY CARBONYL STRETCHING VIBRATIONS

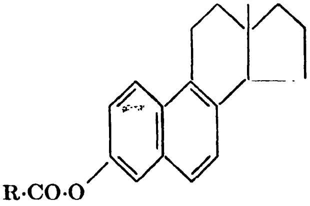
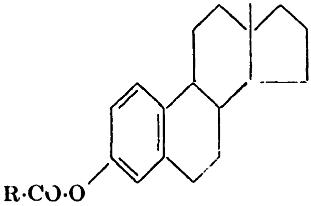
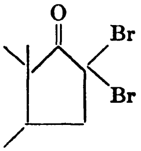
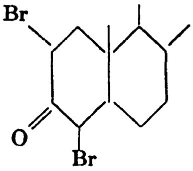
Structure	Frequency of absorption maximum (Carbon disulfide soln.) (cm.$^{-1}$)	Number of compounds examined
 i	1770	1
 ii	1767–1764	4
 iii	1764	1
 iv	1758–1756	4

CHART I.—(*Continued*)

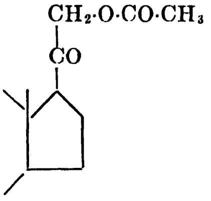
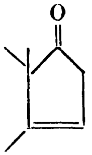
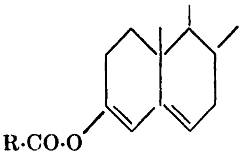
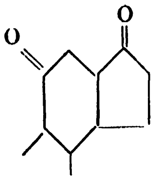
Structure	Frequency of absorption maximum (Carbon disulfide soln.) (cm.$^{-1}$)	Number of compounds examined
 v	1758–1756*	11
 vi	1754	2
 vii	1754	2
 viii	1754*	5

CHART I.—(*Continued*)

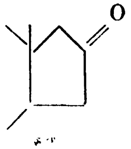
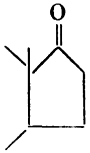
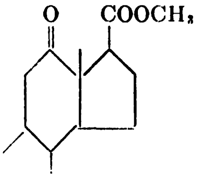
	Structure	Frequency of absorption maximum (Carbon disulfide soln.) (cm.$^{-1}$)	Number of compounds examined
ix		1754–1751	1
x		1745–1742	27
xi	Alkyl esters of cholanic, norcholanic, bisnorcholanic and etiocholanic acids	1742–1737	39
xii		1742–1739*	3
xiii	Acetates and propionates of steroid alcohols	1742–1735	52

CHART I.—(*Continued*)

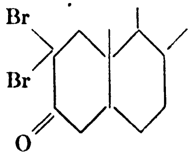
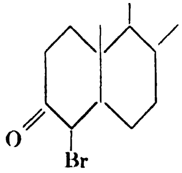
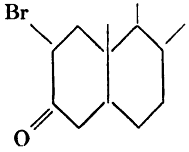
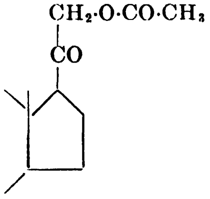
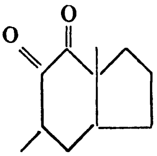
Structure	Frequency of absorption maximum (Carbon disulfide soln.) (cm.$^{-1}$)	Number of compounds examined
 xiv	1737	1
 xv	1735	1
 xvi	1735	3
 v	1732–1729*	11
 xvii	1726	3

CHART I.—(*Continued*)

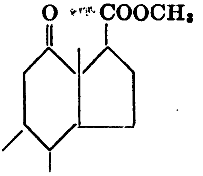
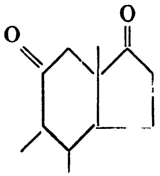
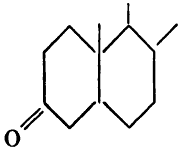
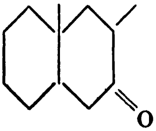
Structure	Frequency of absorption maximum (Carbon disulfide soln.) (cm.$^{-1}$)	Number of compounds examined
Benzoates of steroid alcohols xii	1724–1719	7
 xviii	1723*	3
 viii	1719–1713*	5
 xix	1719–1715	19
 xx	1719–1713	5

CHART I.—(*Continued*)

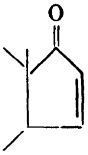
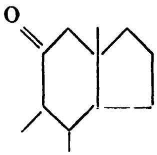
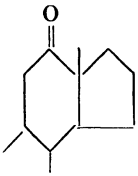
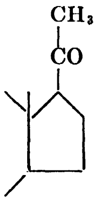
	Structure	Frequency of absorption maximum (Carbon disulfide soln.) (cm.$^{-1}$)	Number of compounds examined
xxi		1716	2
xxii		1716–1710	24
xxiii		1710–1706	3
xxiv		1710–1706	26
xxv	Steroid carboxylic acid	1710–1703	3

CHART I.—(*Continued*)

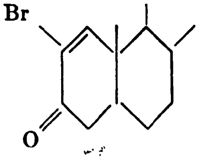
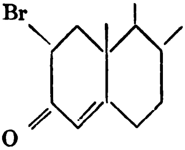
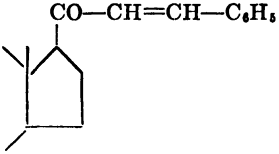
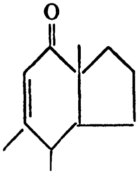
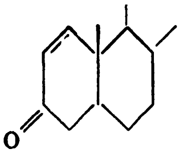
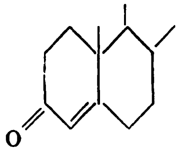
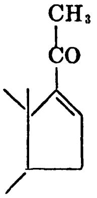
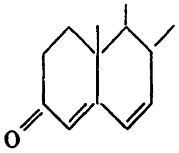
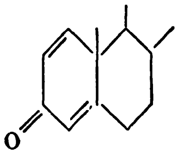
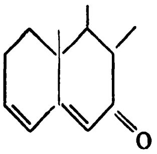
Structure	Frequency of absorption maximum (Carbon disulfide soln.) (cm.$^{-1}$)	Number of compounds examined
 xxvi	1697	3
 xxvii	1697	2
 xxviii	1684	1
 xxix	1684–1680	4
 xxx	1684–1680	4

CHART I.—(*Continued*)

Structure	Frequency of absorption maximum (Carbon disulfide soln.) (cm.$^{-1}$)	Number of compounds examined
 xxxi	1677–1674	20
 xxxii	1670–1666	5
 xxxiii	1669–1666	2
 xxxiv	1666–1663	5
 xxxv	1663	1

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* Two maxima are present, both of which are included in the table.

position of the absorption maximum is shifted by 20–40 cm.$^{-1}$ to lower frequencies (cf. x and xxi; xxiii and xxix; xxiv and xxxii; xix, xxx and xxxi in Chart I). In the *conjugated diene-one systems* the introduction of the second ethylenic double bond produces a smaller additional displacement in the same direction (Chart I, xxxiii, xxxv). These displacements of the band make it easy to distinguish the nonconjugated from the conjugated carbonyl group, and, in many instances, also allow of the distinction between different types of conjugated carbonyl groups. Further information concerning the structure of conjugated carbonyl groups can be derived also from investigation of the C=C stretching vibrations which occur between 1680 and 1580 cm.$^{-1}$ (see page 325).

If the double bond is not conjugated with the carbonyl group, it does not, in general, effect the C=O stretching vibration. An exception has been encountered in the case of the Δ^{14}-17-ketone (Table I, vi) where the introduction of the β,γ-double bond displaces the carbonyl maximum in the opposite direction from 1742 to 1754 cm.$^{-1}$

All data discussed in this section and listed in Chart I were obtained on solutions of the steroid in carbon disulfide. In carbon tetrachloride the maxima occur at close to the same positions, but in chloroform they are displaced to lower frequencies. The magnitude of this solvent effect varies for the different groups, being greatest in the case of the conjugated ketones (Jones *et al.*, 1948c).

Furchgott *et al.* (1946a, b, c, 1947a, b) have examined several ketosteroids as crystalline films deposited from pyridine solution. While the frequencies they report for the maxima agree reasonably with our values in carbon disulfide for the 17-ketone, 20-ketone, Δ^4-3-ketone and the acyl ester carbonyl groups, in other cases (notably the 11-ketone, 3-ketone, 20-keto-21-acetoxy groups, and the 17-ketone in the equilenin and equilin series), there are quite large differences. In our experience the frequency of the carbonyl maximum in steroids measured in the crystalline state in mulls is much more variable than when measured in dilute solution, Haines *et al.* (1948) report that crystalline Δ^5-pregnenol-3 β-one-20 exhibits two maxima at 1700 and 1685 cm.$^{-1}$ In the course of the investigation of about 500 steroids in carbon disulfide solution we have not observed a similar anomaly.

b. Monoesters. The C=O stretching band associated with the carbonyl group of the carboxylic esters occurs between 1745 and 1735 cm.$^{-1}$ and varies very little with the position of the ester group on the steroid molecule (Table I); from the position of this band it is not possible to distinguish between the acetates or propionates of 3, 11, 12, 17, or 20 steroid alcohols. The methyl esters of carboxylic acids in the cholanic, norcholanic, bisnorcholanic and etiocholanic acid series also absorb in the same frequency range. The insensitivity of this vibration to change of position on the ring system is in marked contract to the ketonic carbonyl group discussed above. The difference is probably to be attributed to the fact that in the esters the carbonyl group is separated from the ring system by an intermediate oxygen atom, while in the ketones the carbon atom of the vibrating double bond is an integral part of the steroid structure and is directly subject to the effects of variations in the internal steric and electronic forces in the ring system.

TABLE I

Frequencies of Carbonyl Maxima in Steroid Monoesters
(Solvent Carbon Disulfide)

Type of Ester	Frequency (cm.$^{-1}$)	Number of compounds examined
Phenolic 3-acetate esters (equilenin type)	1770	1
Phenolic 3-acetate esters (equilenin and estrone types)	1767–1764	4
$\Delta^{3,5}$-Diene-ol-3 acetate and propionate enol esters	1754	2
Methyl esters of cholanic, norcholanic, and bisnorcholanic acids	1742	29
17-Acetate and propionate esters	1742–1737	11
Methyl esters of etiocholanic and etioallocholanic acids	1739–1737	10
3-Acetate and propionate esters	1739–1735	35
20-Acetate esters	1735	2
3-Benzoate esters	1724–1719	6

In the benzoates, the carbonyl band is displaced to lower frequencies, as in the conjugated ketones, and no doubt this is a result of the direct conjugation with the benzene ring. In the phenolic acetates and the enol esters the carbonyl absorption is shifted in the opposite direction. In these compounds the carbonyl group is attached to the conjugated system through an ether type oxygen linkage.

c. Carboxylic Acids and δ-Lactones. The examination of the carbonyl group in the free carboxylic acids is handicapped by the low solubility of steroid carboxylic acids in carbon disulfide. Measurements on five compounds indicate that the maximum lies between 1710 and 1700 cm.$^{-1}$ In two steroid δ-lactones which have been examined the maximum is near 1742 cm.$^{-1}$, identical with the position in alkyl esters.*

d. Dicarbonyl and Polycarbonyl Compounds. When two or more carbonyl groups are present together in the molecule three distinct situations can arise.

1. *The groups absorb independently of one another;* the spectrum exhibits a number of maxima equal to the number of carbonyl groups, and the positions of the maxima correspond to those expected of each carbonyl type. Examples of diketones and ketoester systems in which these conditions occur are listed in Table II. This situation is commonly

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* Recent measurements indicate 1780–1777 cm.$^{-1}$ for the carbonyl stretching maximum in the saturated γ-lactone group.

marized in Table III, and curves illustrating the degree of resolution obtained in the diketones are shown in Fig. 6.

These data suggest that under these conditions the two maxima in the 3,20-diketones can be resolved without difficulty.* In the single 11,20-diketone examined the two maxima at 1713 and 1706 cm.$^{-1}$ were not resolved and a single peak at 1709 cm.$^{-1}$ was observed. As yet no suitable 3,11-diketones have been available for study. In pregnanetrione-3,11,20 one maximum is found at 1711.

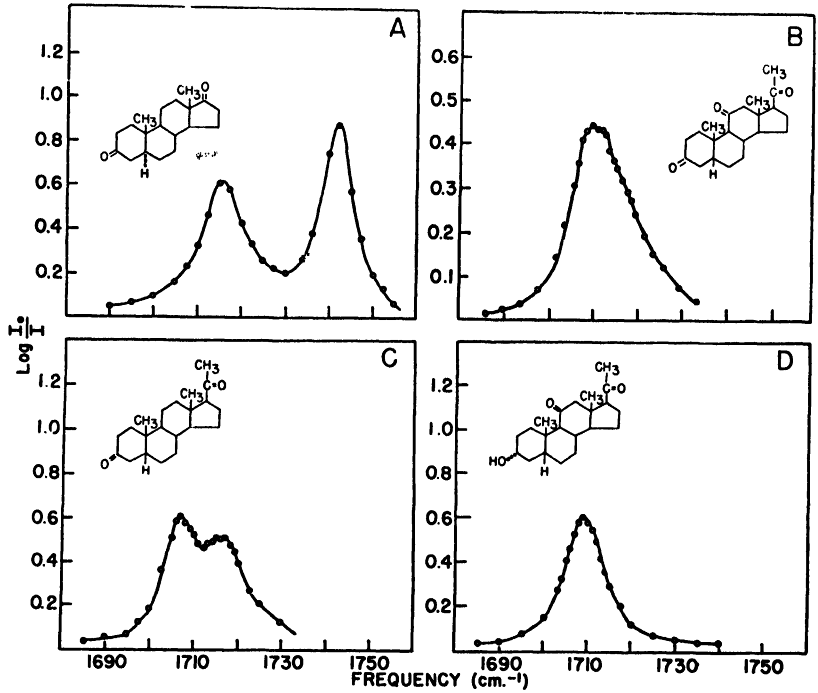

FIG. 6. Spectra of di- and tricarbonyl compounds in the region of the C=O stretching vibration, determined with a calcium fluoride prism (carbon disulfide solution).

In the cases where the two carbonyl maxima are not resolved, the presence of the two carbonyl groups may be demonstrated by measurement of the absolute intensity of the band (page 333).

3. *The number of bands observed corresponds with the number of carbonyl groups present, but the positions of the maxima are displaced.* It is evident that in these cases the carbonyl groups are subject to a mutual

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* 3,17-Diketones can be resolved without difficulty with the sodium chloride prism.

interaction which disturbs their vibration frequencies. The groups in which these conditions have been observed are summarized in Chart II. This interaction phenomenon has so far only been encountered when the two carbonyl groups are in the 1:2, 1:3, or 1:4 positions relative to one another. Thus it is observed in the 12-keto-17-carbomethoxy structure (XXVII) but not in the 11-keto-17-carbomethoxy grouping (XXVIII). Similarly it is seen in the 11,17-diketones (XXIX) but not in the 11,20-diketones (XXX). It may also be significant that where such effects occur the absorption maxima are always displaced to higher frequencies. It is not possible to state whether the displacement results from inter-

TABLE III

Frequencies of Carbonyl Maxima in 3, 11, 17, and 20 Ketosteroids as Determined with a Calcium Fluoride Prism

(Carbon Disulfide Solution)

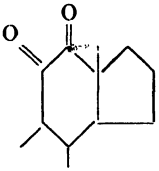
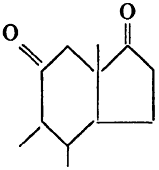
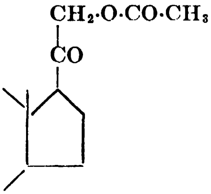
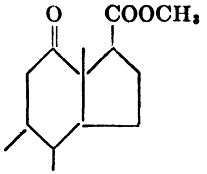
Compound	Frequency
3-Ketone	
Androstanone-3	1715
Allopregnanone-3	1716
Cholestanone-3	1715
11-Ketone	
11-Ketonorcholanic acid methyl ester	1713*
3,9-Epoxy-11-ketonorcholanic acid methyl ester	1713*
3,9-Epoxy-11-ketocholanic acid methyl ester	1713*
17-Ketone	
Androstanol-3 α-one-17	1741
Etiocholanol-3 α-one-17	1742
Δ^4-Androstenol-3 α-one-17	1742
20-Ketone	
Allopregnanol-3 α-one-20	1706
Δ^5-Pregnenol-3 β-one-20	1707
3,17-Diketone	
Androstanedione-3,17	1716, 1742
Etiocholanedione-3,17	1717, 1742
3,20-Diketone	
Allopregnanedione-3,20	1707, 1716
Pregnanedione-3,20	1707, 1716
11,17-Diketone	
See Chart II.	
11,20-Diketone	
Pregnanol-3 α-dione-11,20	1709
3,11,20-Triketone	
Pregnanetrione-3,11,20	1711

* These compounds contain a second band at 1742 associated with the methyl ester carbonyl group.

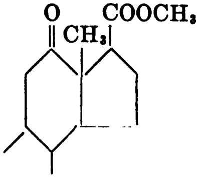
CHART II

Positions of Carbonyl Maxima in Dicarbonyl Compounds Showing Interaction Effects

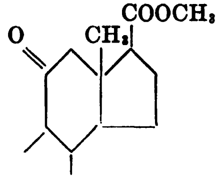
(Carbon Disulfide Solution)

Structure	Predicted position Assuming no interaction	Observed positions	Number of compounds examined
A. *Diketones*			
	1716–1710	1729–1726	3
	1745–1742 1716–1710	1754 1719–1716	4
B. *Ketoesters*			
	1742–1739 1710–1706	1758–1754 1732–1729	5
	1742–1739 1710	1742–1739 1723	3

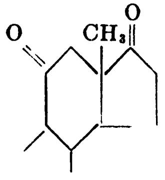
actions occurring by transmission through the intermediate bonds, or as a result of a direct field effect.



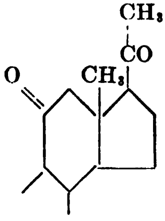
XXVII



XXVIII

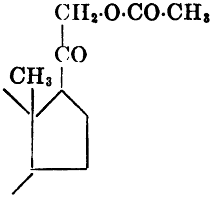


XXIX



XXX

These interaction effects have proved of considerable help in the recognition of certain structural features. The 11,17-diketone structure (XXIX) and the 20-keto-21-acetoxy (XXXI) grouping are particularly important in connection with metabolic studies and are discussed elsewhere (page 357).



XXXI

e. The Recognition of the Acetate Group. From Chart I it will be seen that both the 17-ketone group and the acetate ester group absorb in the same position near 1740 cm.$^{-1}$ In the acetates a second intense band stands out prominently in the region between 1228 and 1245 cm.$^{-1}$; this is lacking from the spectrum of the 17-ketone and readily permits a distinction to be made between the two structures. This band is probably to be associated with a motion of the C—CO—O system of the ester group (Thompson and Torkington, 1945; Elliott *et al.*, 1948). In the 20-keto-21-acetoxy compounds it is displaced from 1240 to 1228–1230 cm.$^{-1}$ (Jones *et al.*, 1948c).

f. Brominated Ketosteroids. Other functional groups in proximity to the carbonyl group may also influence its band position. It is of interest to note, particularly in connection with applications to steroid synthetic work, that some systematic effects of bromine atoms have been observed. Included in Chart I are several structural groups (iii, iv, xiv, xv, xvi, xxvi, xxvii) containing bromine atoms on the carbon atom α to the ketone group (Djerassi and Scholz, 1947; Wilds and Djerassi, 1946).* The 3-ketone band is displaced by 15 cm.$^{-1}$ to higher frequency from its normal position on bromination of the α-carbon atom. This holds both for the nonconjugated and the conjugated 3-ketone. A similar displacement has also been demonstrated in one instance for a nonconjugated ketone group at position 17, brominated at 16. The introduction of a second bromine atom on the same α-carbon atom (Chart I, xvi) has no additional effect, but a second bromine atom at the α-carbon on the opposite side of the carbonyl group (Chart I, iv) causes an additional displacement of 15 cm.$^{-1}$ The introduction of bromine atoms β to the carbonyl group does not displace the band position. The effect of α-bromination on the absolute intensity of the carbonyl absorption is discussed on page 334.

g. Discussion. The frequency shift of the absorption maximum in these carbonyl compounds is a measure of the change of force constant of the C=O bond, and an increase in the frequency of the band indicates an increase in the force constant. This may be expected to show a parallel effect both on the strength of the double bond, as indicated by its chemical reactivity and on the mean length of the bond as determined by electron and X-ray diffraction studies. The correlation of such physical and chemical properties of the carbonyl group offers an interesting field of research (see Flett, 1948).

2. The Absolute Intensities of Carbonyl Bands

a. Methods. In the application of *ultraviolet* spectrometry to the elucidation of molecular structure, it has been customary for many years to determine the intensity of the absorption bands, as well as the frequency or wavelength of the absorption maxima. The intensity is expressed as the molecular extinction coefficient at the absorption maximum (ϵ).

$$\epsilon_{max} = \frac{1}{cl}\left(\log_{10}\frac{I_0}{I}\right)_{max} \qquad \text{(i)}$$

where c is the molar concentration of the solute, l is the cell length in centimeters and $(\log I_0/I)_{max}$ the optical density at the absorption maximum.

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* We wish to thank Dr. Wilds (University of Wisconsin) and Drs. Djerassi and Scholz (Ciba Pharmaceutical Products Inc.) for making this series of compounds available to us.

In *infrared* spectrometry, it is only recently that attention has been given to the measurement of absolute intensities. This is a consequence of certain experimental difficulties which are encountered.

In the derivation of the expression for the molecular extinction coefficient (equation i) it is assumed that the optical density is determined with pure monochromatic radiation. This is never true in practice, but provided the slit-width is small in comparison with the half-width of the band, the deviation from strict monochromaticity will not appreciably alter the observed value of ϵ_{max}. Under the conditions normally employed in *ultraviolet* spectrometry this requirement is met, the slit-width to half-band width ratio being seldom greater than 0.2 and usually much less. In the infrared the absorption bands are relatively narrower and ratios of 0.5 to 1.0 are commonly used. Because of this, the experimentally observed values of ϵ_{max} will vary with the slit-width, the shape of the absorption band and the resolving power of the spectrometer.

Most of the experimental work which has been carried out on the determination of the absolute intensity of infrared absorption bands has been in connection with theoretical studies of the absorption spectra of simple compounds, mainly gases (Wilson and Wells, 1946; Thorndike *et al.*, 1947). The integrated absorption intensity defined by (ii) where,

$$A = \frac{1}{cl} \int \log_e \left(\frac{I_0}{I}\right) d\nu \tag{ii}$$

(the integral is taken over the limits of the absorption band) has been measured rather than ϵ_{max} since this function has theoretical significance and can be related to the dipole moment of the molecule. Furthermore, as is shown below, the integrated absorption intensity can be evaluated from measurements with finite slits.

Since A involves $\left(\frac{I_0}{I}\right)$ it will be subject to the errors resultant from the use of finite slits, but Wilson and Wells (1946) have carried out a mathematical analysis of the effect of the slit width and shown that if A' is the apparent integrated absorption intensity determined with a finite slit, the true integrated absorption intensity A may be derived by measuring A' for a series of different concentrations using a fixed slit width and extrapolating to zero concentration.

An example of such a plot for the carbonyl band of a ketosteroid measured in carbon tetrachloride solution is shown in Fig. 7. The absolute intensity of the carbonyl band is given by the intercept of the line with the y-axis.

Although the absolute intensities of the carbonyl stretching bands in several steroids have been obtained in this manner, the measurements are involved and tedious. The absorption spectrum must be determined quantitatively over the region of the band at three or four concentrations. To evaluate A' at each concentration, the optical densities must be measured at several frequencies, plotted against the frequency, and the area under this curve then integrated. The value of A' so obtained for each concentration is next plotted against the concentration and the resultant graph extrapolated to zero concentration.

Methods by which this procedure can be considerably simplified have been developed recently by Dr. D. A. Ramsay (Jones *et al.*, 1948b) in the Ottawa laboratories. He has shown that, in the monocarbonyl compounds, the carbonyl absorption band can be fitted closely by equation (iii).

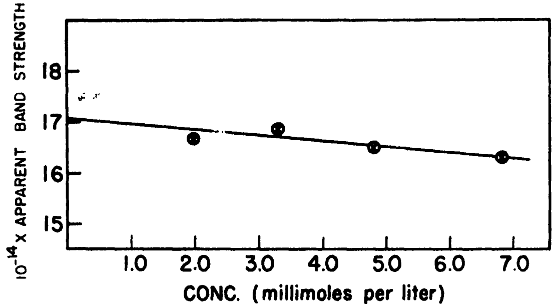


FIG. 7. Plot of apparent band strength (A′) against concentration for the carbonyl stretching bands in progesterone (carbon disulfide solution).

$$2.303 \log_{10} \frac{I_0}{I} = \frac{a}{(\nu - \nu_0)^2 + b^2} \tag{iii}$$

in which ν_0 = frequency at the absorption maximum and a and b are constants. From this equation it may be shown that the apparent integrated absorption intensity A' is given by (equation iv)

$$A' = 2.303 \frac{\pi}{2} \epsilon_{\text{max}}' \delta_{\frac{1}{2}} \tag{iv}$$

in which ϵ_{max}' is the apparent value of the molecular extinction coefficient determined with a finite slit, and $\delta_{\frac{1}{2}}$ the band width at half the maximum intensity, using the same slit width.

Where two or more overlapping carbonyl absorption bands occur, as in most of the diketones, this method of evaluating A cannot be used and it is necessary to resort to the graphical integration method.

b. Absolute Intensities of Individual Carbonyl Maxima. In Table IV the results of some measurements on the integrated absorption intensities of some steroid monocarbonyl compounds are listed. Although the data available are considerably fewer than those on which the correlations between the frequency of the maxima and chemical structures were established, certain definite regularities in the intensities are apparent.

For a given position of substitution, the ketone band intensity has a constant value. This is lowest for the nonconjugated 20-ketone (5.2–5.5) and highest for the conjugated 3-ketone (11.0–12.1). The values for the nonconjugated 3-ketone (7.9) and the 17-ketone (7.6–8.1) are intermediate and closely similar. It will be noted that the order of increasing intensity in these bands does not parallel the order of increasing frequency of the maxima. Also included in Table IV is the value of 9.2–9.4 units for the steroid monoacetate group.

TABLE IV

Absolute Intensities of Carbonyl Bands in Steroid Monocarbonyl Derivatives

Compound	Integrated absorption intensity $\times 10^{-14}$ cycles/ mole^{-1}/liter/ cm.$^{-1}$	Solvent
3-Ketone		
Androstanol 17 α-one-3 (*Dihydrotestosterone*)	7.9	CS_2
Cholestanone-3	7.9	CCl_4
Δ^4-3-Ketone		
Δ^4-Androstenol-17 α-one-3 (*Testosterone*)	12.1	CCl_4
Δ^4-Androstenone-3	11.0	CS_2
17-Ketone		
Δ^5-Androstenol-3 β-one-17 (*Dehydroisoandrosterone*)	7.9	CCl_4
Androstanol-3 α-one-17 (*Androsterone*)	7.6	CCl_4
Etiocholanol-3 α-one-17	8.1	CCl_4
Monoacetate Ester		
Cholestanol-3 β-acetate	9.4	CCl_4
Cholestanol-3 β-acetate	9.2	CS_2
20-Ketone		
Pregnanol-3 α-one-20	5.2	CCl_4
Δ^5-Pregnenol-3 α-one-20	5.5	CCl_4
α-Brominated 3-Ketone		
2-Bromocholestanone-3	5.6	CS_2
2,4-Dibromcholestanone-3	3.4	CS_2

In Table V the experimental values of the Integrated absorption intensity obtained by graphical integration for a variety of dicarbonyl compounds are compared with those predicted on the assumption that the intensities of the individual carbonyl maxima may be added arithmetically. In all cases a fairly good agreement is observed. In the di- and triacetates, the additivity of the acetate intensities are clearly demonstrated. Similar agreement has been observed also for the acetate bands at 1240 cm.$^{-1}$ (see page 329) but in that case overlapping with adjacent bands introduces uncertainties in the evaluation. From the figure of 18.3 for

3-acetoxycholanic acid methyl ester, a value of 18.3 − 9.3 = 9.0 is arrived at for the cholanic acid methyl ester group. The introduction of bromine atoms in the α-position appear to diminish the intensity of absorption (cf. page 330).

The measurements of the absolute intensity of carbonyl groups which have been made so far are largely of a preliminary nature, but it is apparent that information of considerable value in the elucidation of steroid structure is to be derived from them and further investigations along these lines are underway.

TABLE V

Absolute Intensities of Carbonyl Bands in Di- and Tricarbonyl Compounds

Compound	Integrated absorption intensity × 10^{-14} (cycles/mole/liter cm.) Observed	Calc. from data in Table IV	Solvent
3,17-Diketone			
Androstanedione-3,17	15.8	7.9 + 7.8 = 15.7	CS_2
3,20-Diketone			
Allopregnanedione-3,20	13.6	7.9 + 5.3 = 13.2	CS_2
Δ⁴-3,20-Diketone			
Δ^4-Pregnenedione-3,20 (*Progesterone*)	17.5	11.6 + 5.3 = 16.9	CS_2
11,20-Diketone			
Pregnanol-3α-dione-11,20	10.4		CS_2
17-Ketoacetate			
Androstanol-3α-one-17-acetate (*Androsterone acetate*)	17.3	7.9 + 9.3 = 17.2	CS_2
20-Keto-21-Acetate			
Pregnanediol-3α-21-one-20-acetate-21	14.6	5.4 + 9.3 = 14.7	$CHCl_3$
Diacetate			
Pregnanediol-3α,20β-diacetate	18.6	9.3 × 2 = 18.6	CS_2
Triacetate			
Allopregnanetriol-3α,16,20-triacetate	27.0	9.3 × 3 = 27.9	CS_2
Acetoxycholanic acid Ester			
3α-Acetoxycholanic acid methyl ester	18.3		CS_2

3. The Hydroxyl Group

One or more hydroxyl groups are often present in naturally occurring steroids, and reference to the spectrum of androsterone in Fig. 2 will show that in dilute solution in carbon disulfide there is a sharp band at the extreme high frequency end of the spectrum (3600 cm.$^{-1}$) which is due to the stretching of the O—H bond and characteristic of the hydroxyl group. This band is invariably seen when an unassociated hydroxyl group is present. Occasionally in solution, and very frequently in the crystalline state, the appearance of this hydroxyl band is greatly changed as a consequence of the effects of hydrogen bonding. It becomes broadened, intensified, and displaced to lower frequencies. In contrast to the carbonyl absorption bands the position of this hydroxyl band does not appear to show any regular displacement with position on the ring system.

4. The Ethylenic Double Bond

Three types of carbon-carbon double bonds are encountered in naturally occurring steroids: (*a*) single ethylenic double bonds (*b*) conjugated double bonds, and (*c*) aromatic ring systems.

Very frequently in the spectra of compounds containing any of these types of double bond a characteristic band can be detected near 3000 cm.$^{-1}$ This may appear either as a distinct band or as an inflection on the high frequency side of the intense group of bands associated with the —C—H stretching vibration. This band is in fact a modified C—H stretching vibration and is produced by the C=C—H group (Fox and Martin, 1940); an example is given in Fig. 8 (curve C).

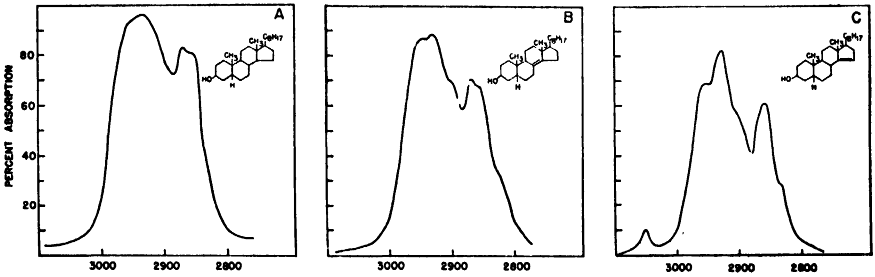
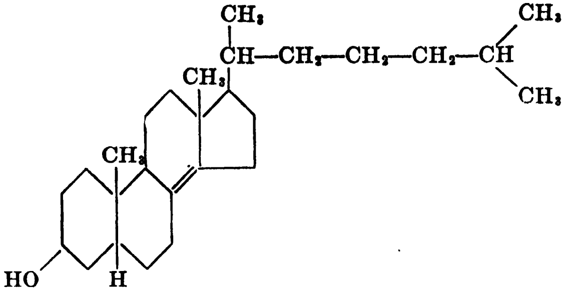


Fig. 8. Region of the C—H stretching vibrations in the spectra of saturated and unsaturated steroids (carbon tetrachloride solution).

In our experience this band is not always a reliable criterion of unsaturation, as it will not be observed when the double bond has no attached hydrogen as in XXXII (see Fig. 8, curve B).

The stretching vibration of the —C=C— group occurs between 1670 and 1580 cm.$^{-1}$ In compounds containing conjugated dienes and in α,β-unsaturated ketones, strong bands are seen in this region. The positions of the bands are characteristic of specific structures as indicated in Chart III. If the double bond is not conjugated, the absorption associated with the C=C stretching vibration is often of very low intens-



XXXII

ity. For certain types of double bonds, such as Δ^4 and $\Delta^{9:11}$, the bands are exceedingly weak and cannot always be detected with certainty.

In this region of the spectrum carbon disulfide absorbs strongly and the spectra are best determined in chloroform solution. Many unsaturated steroids also contain carbonyl groups; the carbonyl absorption band is of very much greater intensity and in some instances the weak double bond absorption becomes obscured by the overlap of the strong carbonyl band. A further technical difficulty arises in the detection of this band due to the strong absorption of atmospheric water vapor in this region of the spectrum and great care is needed to correct for this absorption.

5. Other Structural Correlations

The C—H stretching vibrations which give rise to at least two peaks in the region between 3200 and 2800 cm.$^{-1}$ (Fox and Martin, 1938) are common to most organic compounds. The displacement of these bands in the deuterium-enriched steroids has been discussed previously (page 311). In the region between 1350 and 1480 cm.$^{-1}$ bands occur which can be assigned to bending vibrations of C—H bonds. Bands between 1410 and 1480 cm.$^{-1}$ are associated with vibrations of methylene groups (Rasmussen, 1948; Sheppard, 1948) while methyl group vibrations occur between 1350 and 1400 cm.$^{-1}$ A comparative study of carbon tetrachloride solutions of a variety of steroids suggests that when the methyl or methylene group is adjacent to a carbonyl group these bands are displaced to lower frequencies in a characteristic manner.

6. Discussion

The attempt described here to correlate the position of the absorption bands with molecular structure has provided pertinent information concerning the presence or absence of certain functional groups in the steroid molecule. In the case of the ketonic carbonyl group and the ethylenic double bond it has permitted the position of the group to be located on the steroid nucleus. The presence of hydroxyl and carboxylic ester groups can be detected but as yet their location in the molecule cannot be determined.

There are many important features of steroid structure about which infrared spectrometry so far has provided no information. It is noteworthy to observe that by inspection of the spectrum it is not even possible to state whether a given compound is or is not a steroid. The discussion of steroid structure developed in the foregoing pages has been concerned almost exclusively with the high frequency region (3600–1200 cm.$^{-1}$), but it must be kept in mind that structural variations, such as the introduction of a double bond, also induce large changes in the shape of the curve at frequencies below 1200 cm.$^{-1}$ (Fig. 4). Modifications are produced also by stereochemical inversions (Fig. 3). Studies at the

CHART III

FREQUENCY OF $C=C$ STRETCHING BAND IN UNSATURATED STEROIDS

(Chloroform Solution)

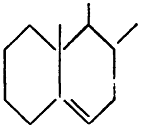
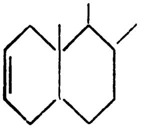
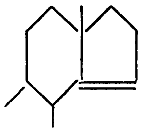
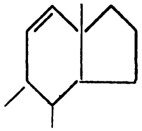
Structure	Frequency (cm.$^{-1}$)	Intensity*	Number of Compounds
A. *Nonconjugated Double Bonds*			
i 	1672–1669	x	10
ii 	1657–1654	xx	2
iii 	1648–1646	x	4
iv 	1628–1625	x	4

CHART III.—(*Continued*)

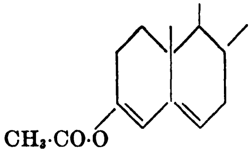
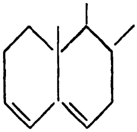
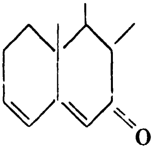
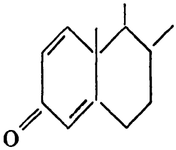
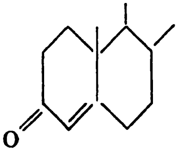
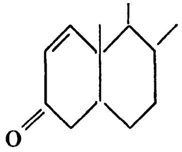
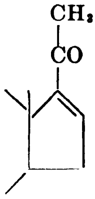
Structure	Frequency (cm.$^{-1}$)	Intensity*	Number of Compounds
B. *Conjugated Dienes*			
 v	1671–1670 1639	xxx	2
 vi	1618 1578	xxx	1
C. *Conjugated Unsaturated Ketones*			
 vii	1627 1598	xxx	1
 viii	1621 1606–1603	xxx	5
 ix	1619–1615	xxx	9

CHART III.—(*Continued*)

	Structure	Frequency (cm.$^{-1}$)	Intensity*	Number of Compounds
x		1609–1600	xx	5
xi		1590–1588	xxx	5

* xxx (strong); xx (medium); x (weak).

higher frequencies have given no indication of spectral variations which can be related to the stereochemistry of the molecule. This is an important problem in the elucidation of steroid structure, concerning which infrared spectrometry has so far failed to make a significant contribution.

The absorption in the 1200–600 cm.$^{-1}$ region is associated with carbon hydrogen bending motions (Sheppard, 1948; Rasmussen, 1948), and with stretching vibrations involving singly bonded carbon and oxygen atoms. Its theoretical interpretation is a matter of extreme difficulty, but nevertheless, it is in this part of the spectrum that absorption characteristic of specific stereochemical configurations is most likely to be observed. Furchgott *et al.* (1948b, c, 1947a, b) have suggested certain relationships between specific stereochemical configurations and absorption bands in this region of the spectrum. We also have observed that absorption near 1000 cm.$^{-1}$ is particularly susceptible to structural and stereochemical changes involving the asymmetric carbon atoms at 3 and 5, but we have as yet been unable to establish any specific correlations which are of general application.

VI. APPLICATIONS TO THE INVESTIGATION OF STEROID METABOLITES IN URINE

The reasons which prompted these studies of the infrared absorption spectra of steroids were mentioned briefly in the introductory paragraphs of this article.

By purely chemical methods (Dobriner *et al.*, 1948b) it had proved impossible to detect and identify *all* of the steroid metabolites excreted in urine. An analytical technique, based on infrared spectrometry, has been developed which has made possible the detection and characterization of (*a*) previously known steroids, (*b*) mixtures of known steroids, and (*c*) new steroids (Dobriner *et al.*, 1948d).

In the case of new steroids, infrared spectrometry has yielded important information about the structure of the compounds. This application is of great value, particularly where the compounds are obtained in very small amounts. The chemical identification of the compounds is considerably aided by the information derived from the infrared spectrum, and in some instances this has been achieved initially by infrared spectrometry alone and only confirmed chemically at a later date when greater quantities have become available (Lieberman and Dobriner, 1949a, Lieberman *et al.*, 1949b).

The infrared spectrum is a much more specific means for the characterization of steroids than the melting point, mixed melting point and optical rotatory power, and information gained and techniques developed in connection with the isolation of steroids from natural sources have been applied also in the field of steroid synthesis (Lieberman *et al.*, 1948c). The use of infrared spectrometry in the recognition of steroids labelled with deuterium has wide application in clinical studies (Dobriner *et al.*, 1949b).

1. Qualitative Applications

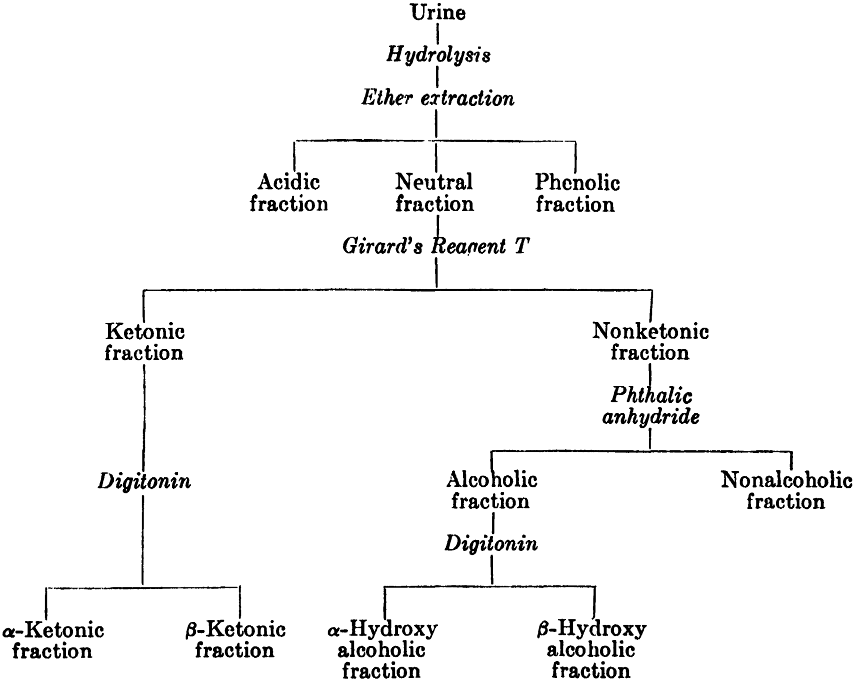
a. Individual Compounds. The methods developed for the isolation of urinary steroids have been discussed in detail elsewhere (Dobriner *et al.*, 1948b), and a brief outline only will be attempted here. After extraction from the urine the steroids are separated by chemical means into a series of primary fractions (see Chart IV). Each of these fractions consists of a complex mixture of steroids and is next submitted to a procedure of chromatographic separation. This chromatographic adsorption process is performed under carefully controlled and standardized conditions and yields several hundred fractions.

The infrared absorption spectra of all the eluted chromatogram fractions are determined over the fingerprint region from 1200 to 800 cm.$^{-1}$ The instrumental conditions are standardized and the instrumental records, as directly obtained for the fractions are compared with standard curves obtained in a similar manner for known pure steroids.

An example is given in Fig. 9 where the instrumental record of a fraction obtained by chromatographic analysis of the α-ketosteroid mixture from humane urine is compared with a standard curve of a sample of pure androsterone. The similarity of the two curves is evident.* In this instance the urinary fraction was not crystalline. Under these conditions the relative intensities of the bands in the spectrum of the urine fraction may differ slightly from those in the spectrum of the standard. The identification is based primarily on the identity of the band frequencies and only secondarily on the relative intensities.

CHART IV

Flow Sheet for Extraction and Separation of Steroids from Urine into Primary Fractions



It was established early in the development of this work that, under the standardized conditions of chromatography employed, the substances are always eluted in a definite order. This greatly simplifies the spectrographic analysis. This is illustrated in Fig. 10 where the instrumental records of the spectra of urinary constituents are shown in their sequence of elution from the chromatographic column.

---

* The curves in Figs. 9–12 are direct reproductions of the instrumental records. The bands at the right hand end in Figs. 9, 11, and 12 are calibration bands provided by an external standard of acetone vapor, and permit the curves to be accurately aligned. In these records the absorption intensity increases downwards so the spectra are inverted in comparison with the per cent-absorption curves shown in Fig. 2 and elsewhere. The frequency decreases from right to left.

b. Recognition and Separation of Mixtures. In actual practice a sharp and complete separation of any pair of successively eluted steroids is rarely accomplished in a single chromatogram. The separations may be achieved in many cases by repeated chromatograms, but in some instances only a partial separation of the steroids can be obtained. This is true particularly for steroids of closely similar chemical structure such as the following pairs:

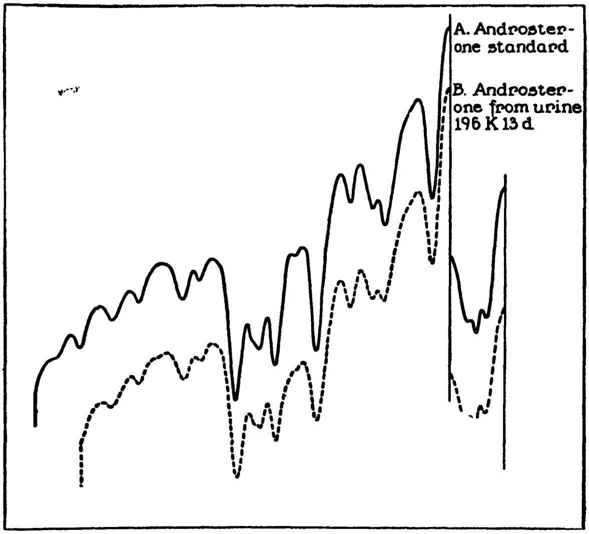


FIG. 9. Comparison of instrumental curve of androsterone isolated from human urine with standard curve.

Androstanol-3 α-one-17 and $\Delta^{9:11}$-Androstenol-3 α-one-17
(*Androsterone*) ($\Delta^{9:11}$-*Androstenolone*)
Etiocholanol-3 α-one-17 and $\Delta^{9:11}$-Etiocholenol-3 α-one-17
(*Etiocholanolone*) ($\Delta^{9:11}$-*Etiocholenolone*)
Androstanol-3 β-one-17 and Δ^5-Androstenol-3 β-one-17
(*Isoandrosterone*) (*Dehydroisoandrosterone*)

For the analysis of such incompletely separable mixtures infrared spectrometry is especially valuable. Standard spectra can be established for known mixtures of the two constituents in varying proportions (Fig. 11). The spectra of the chromatographic eluates may be compared directly with these curves and the composition of the mixture rapidly estimated. The accuracy obtained in such a comparative method

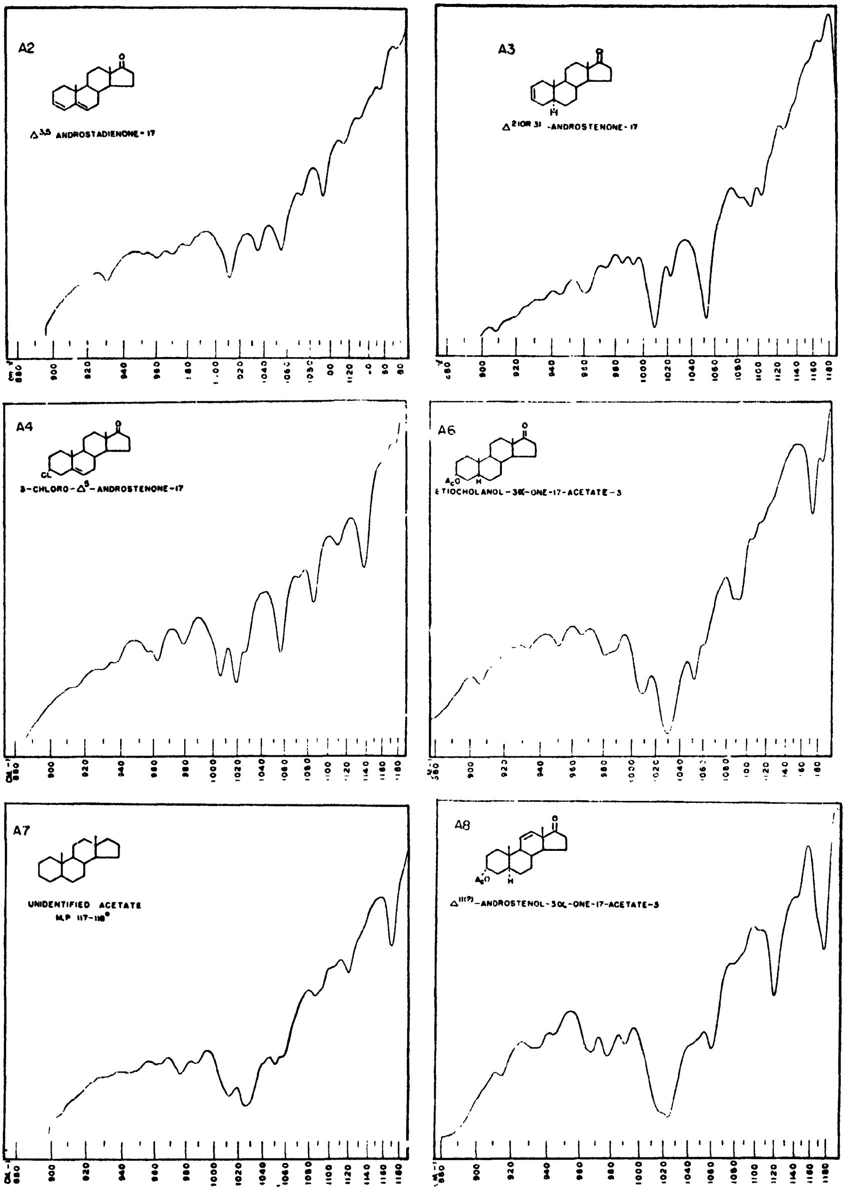


FIG. 10a.

FIGS. 10a–10g. Instrumental curves of carbon disulfide solutions of compounds isolated from urine.

The compounds are arranged in the sequence of their elution under the standard procedures of chromatographic fractionation employed. The codification numbers A2, A3, etc., in the top left hand corner of each diagram relate to Tables I and II of

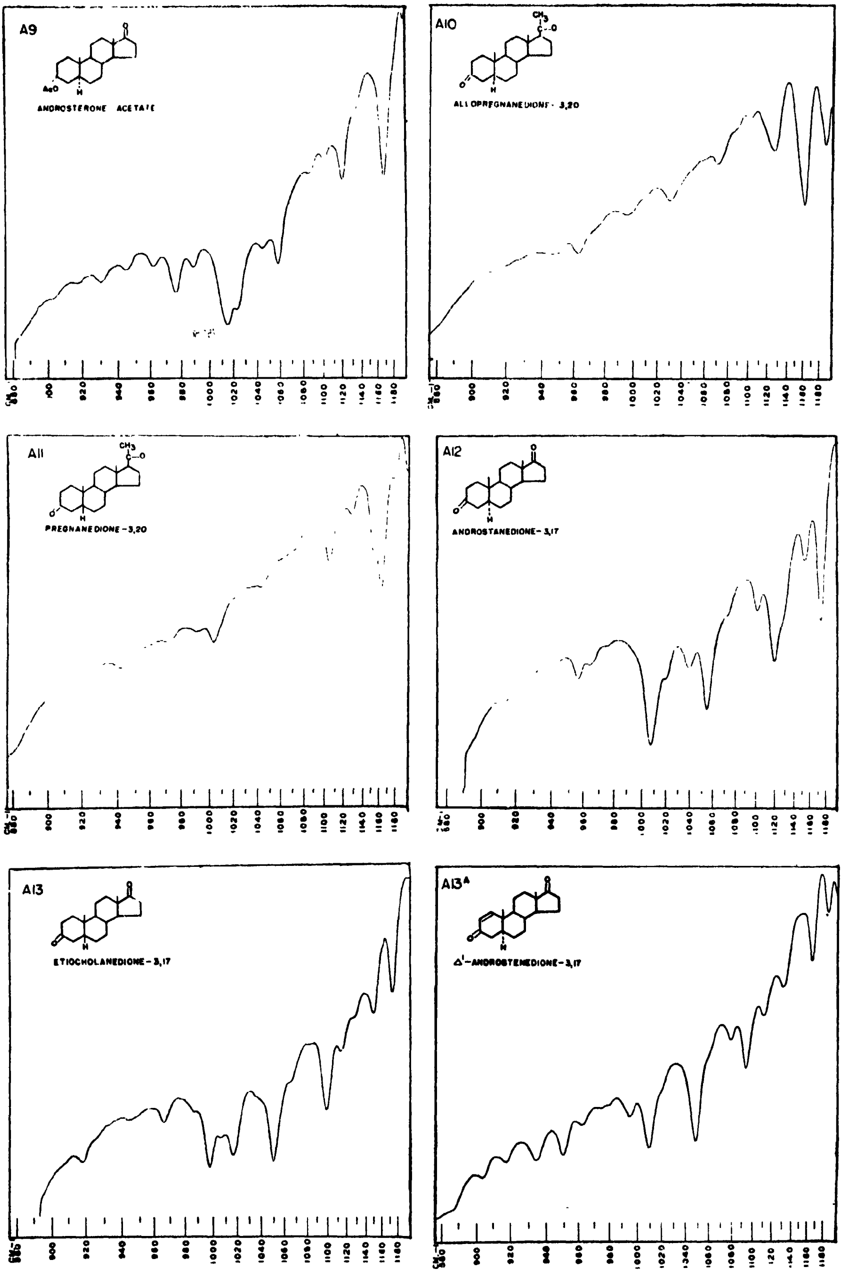

A9
ANDROSTERONE ACETATE
A10
ALLOPREGNANEDIONE-3,20
A11
PREGNANEDIONE-3,20
A12
ANDROSTANEDIONE-3,17
A13
ETIOCHOLANEDIONE-3,17
A13A
Δ¹-ANDROSTENEDIONE-3,17
CM.⁻¹ 880
900
920
940
960
980
1000
1020
1040
1060
1080
1100
1120
1140
1160
1180

FIG. 10b.

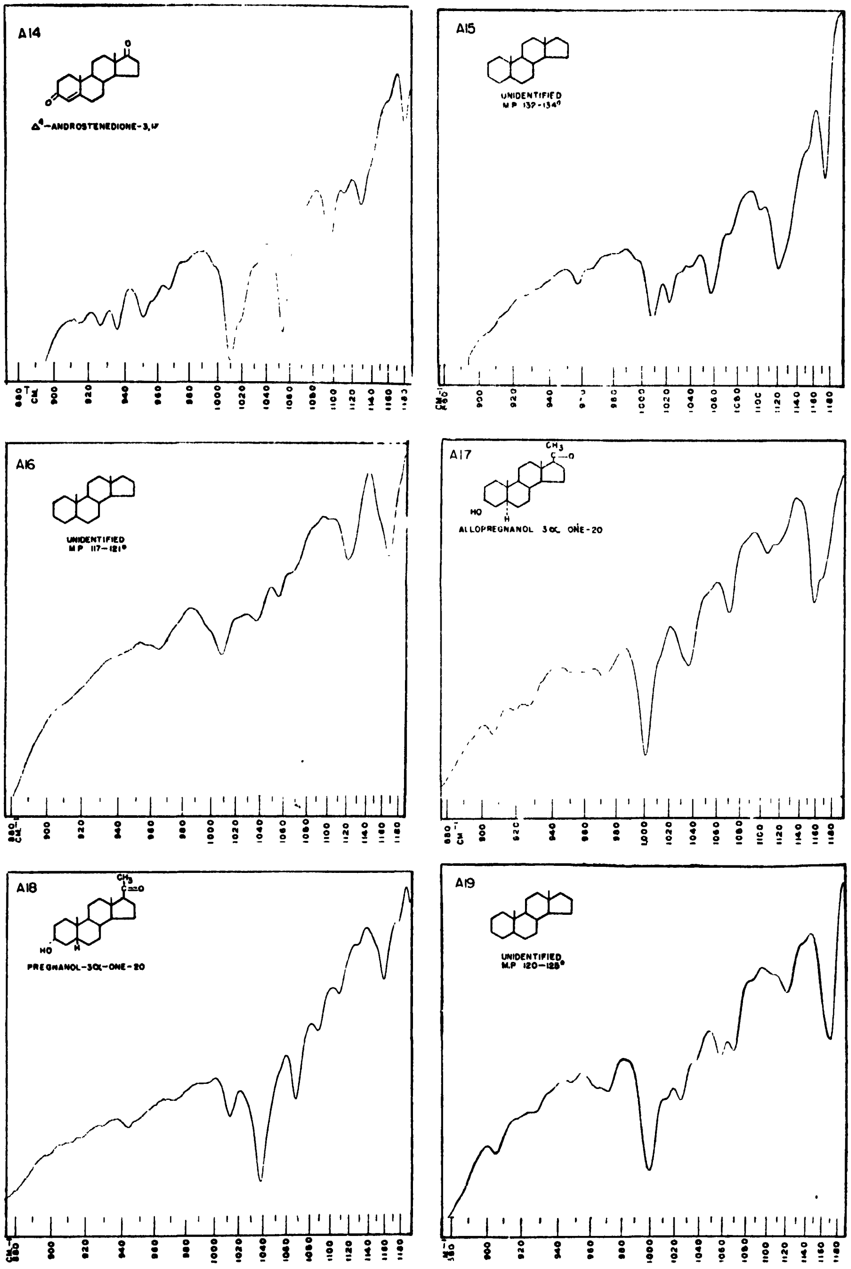

A14
Δ4-ANDROSTENEDIONE-3,17
A15
UNIDENTIFIED
MP 132-134°
A16
UNIDENTIFIED
MP 117-121°
A17
ALLOPREGNANOL 3α ONE-20
A18
PREGNANOL-3α-ONE-20
A19
UNIDENTIFIED
M.P 120-128°
CM.-1
880
900
920
940
960
980
1000
1020
1040
1060
1080
1100
1120
1140
1160
1180

FIG. 10c.

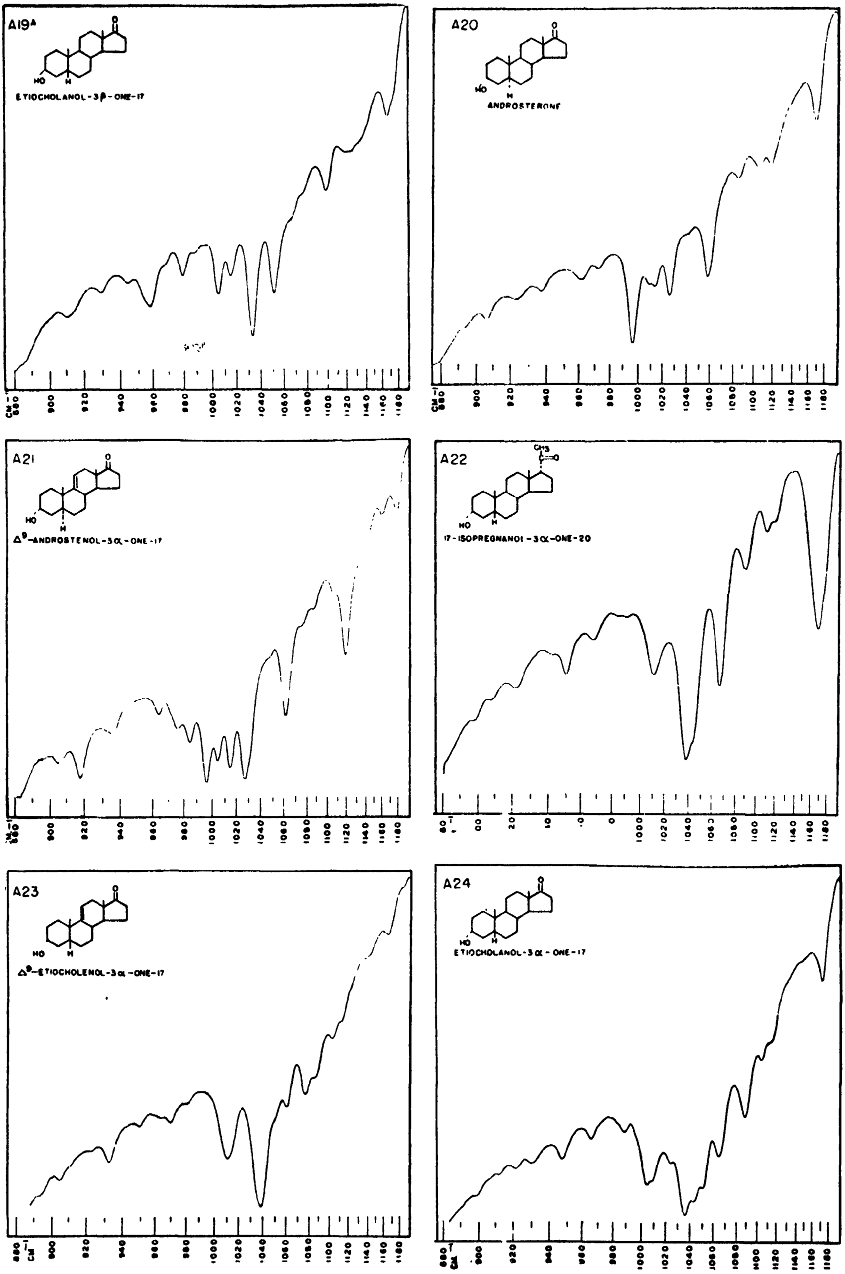

A19a
ETIOCHOLANOL-3β-ONE-17
A20
ANDROSTERONE
A21
Δ9-ANDROSTENOL-3α-ONE-17
A22
17-ISOPREGNANOL-3α-ONE-20
A23
Δ9-ETIOCHOLENOL-3α-ONE-17
A24
ETIOCHOLANOL-3α-ONE-17
HO
H
O
CH3
CM-1
880
900
920
940
960
980
1000
1020
1040
1060
1080
1100
1120
1140
1160
1180

FIG. 10d.

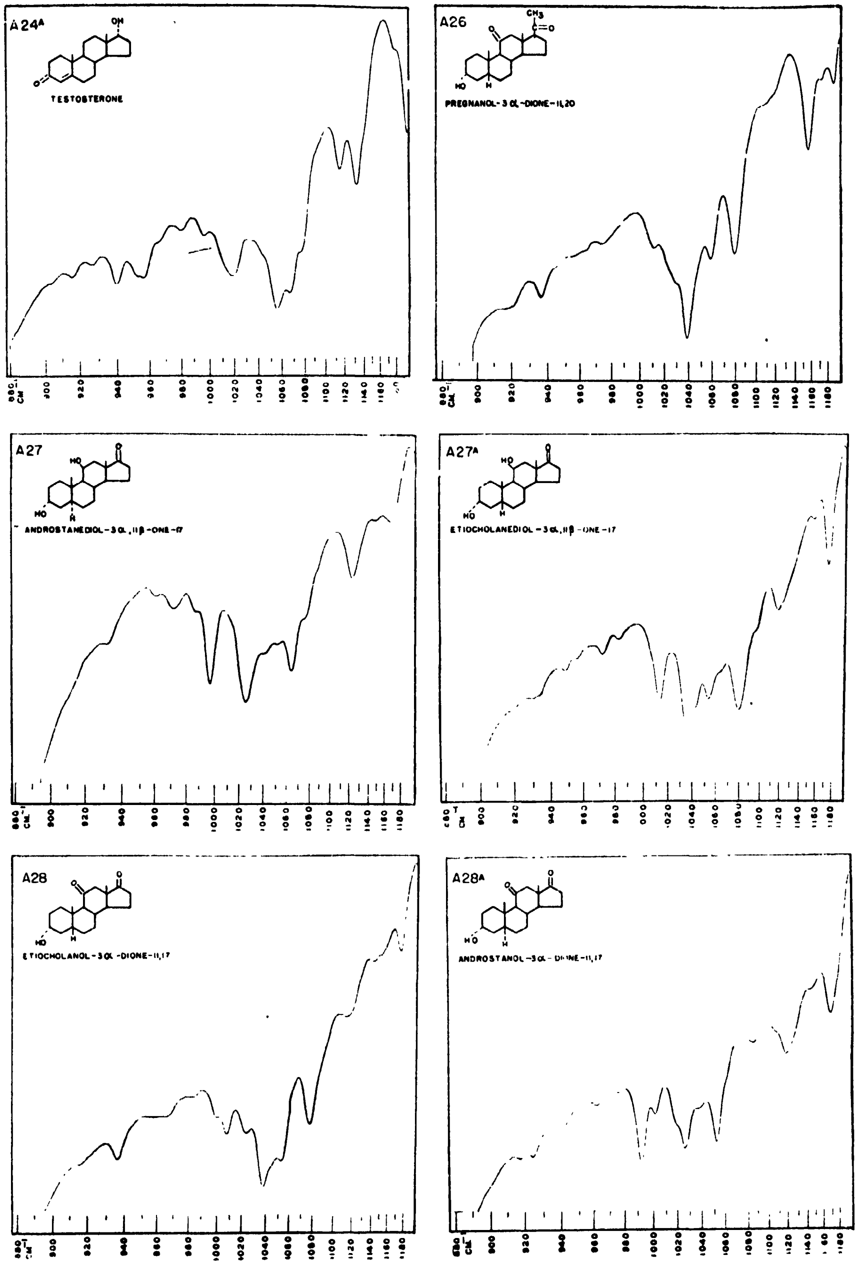

A24A
TESTOSTERONE
A26
PREGNANOL-3α-DIONE-11,20
A27
ANDROSTANEDIOL-3α,11β-ONE-17
A27A
ETIOCHOLANEDIOL-3α,11β-ONE-17
A28
ETIOCHOLANOL-3α-DIONE-11,17
A28A
ANDROSTANOL-3α-DIONE-11,17
880 CM⁻¹
900
920
940
960
980
1000
1020
1040
1060
1080
1100
1120
1140
1160
1180

FIG. 10e.

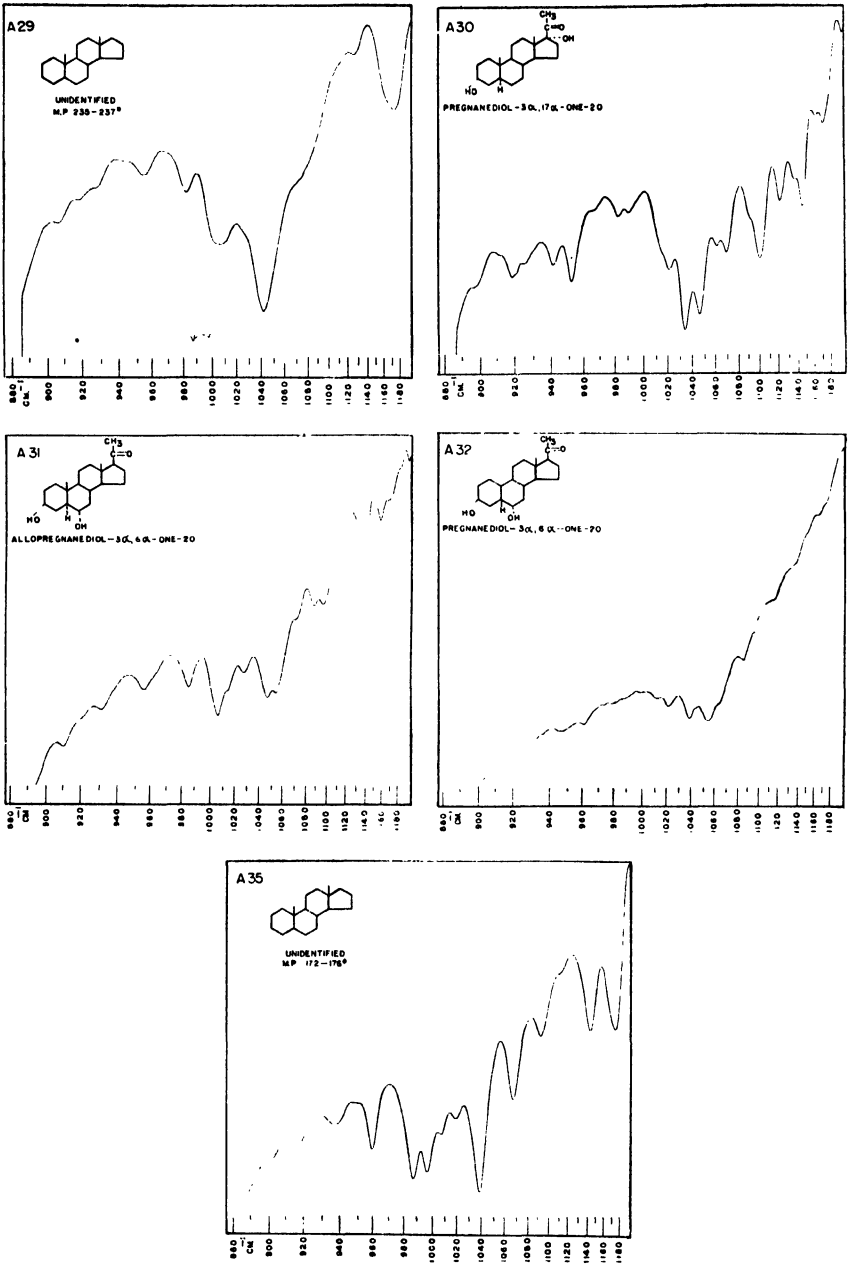

A29
UNIDENTIFIED
M.P 235-237°
A30
CH3
C=O
OH
HO
H
PREGNANEDIOL-3α,17α-ONE-20
A31
CH3
C=O
HO
H
OH
ALLOPREGNANEDIOL-3α,6α-ONE-20
A32
CH3
C=O
HO
H
OH
PREGNANEDIOL-3α,6α-ONE-20
A35
UNIDENTIFIED
M.P 172-176°
880 CM⁻¹
900
920
940
960
980
1000
1020
1040
1060
1080
1100
1120
1140
1160
1180

FIG. 10f.

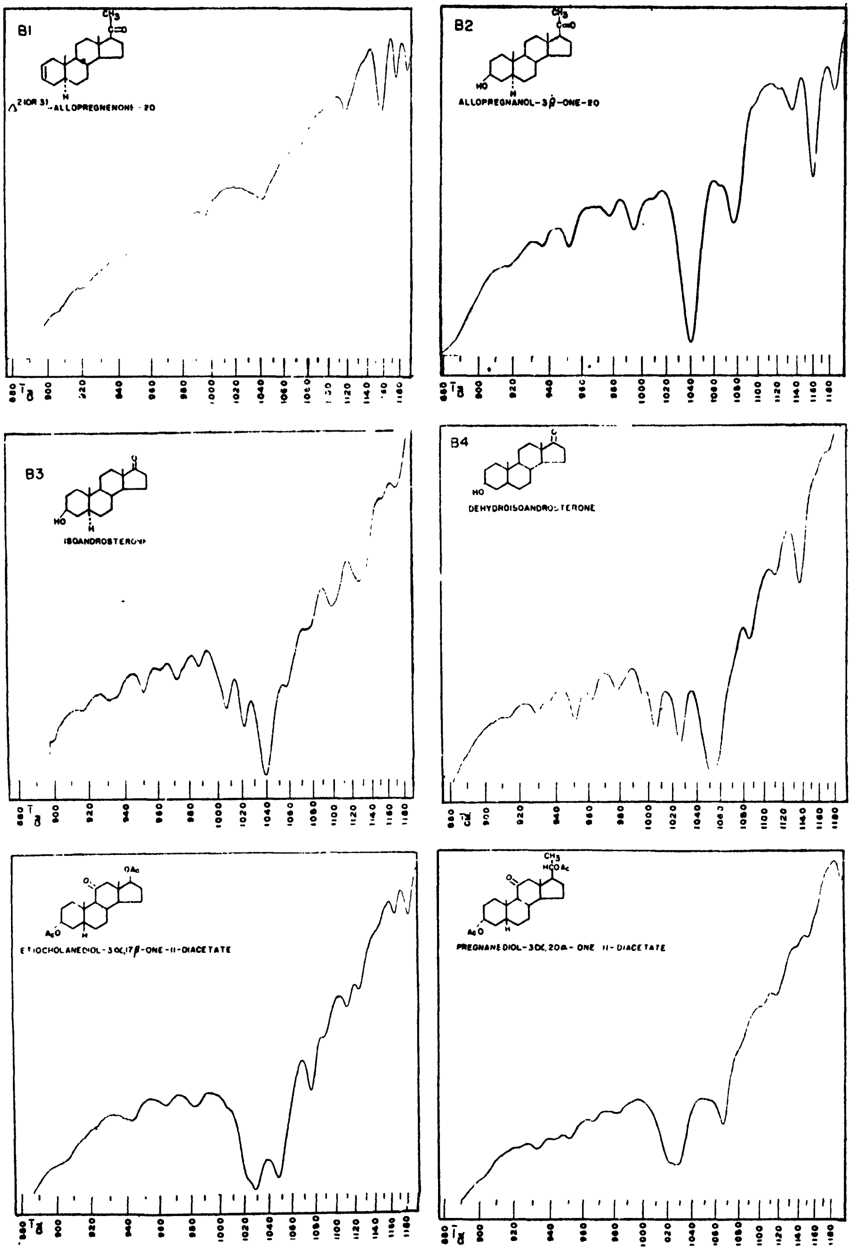

B1
Δ^{2 (OR 3)}-ALLOPREGNENONE-20
B2
ALLOPREGNANOL-3β-ONE-20
B3
ISOANDROSTERONE
B4
DEHYDROISOANDROSTERONE
ETIOCHOLANEDIOL-3α,17β-ONE-11-DIACETATE
PREGNANEDIOL-3α,20α-ONE 11-DIACETATE
880 CM⁻¹ 900 920 940 960 980 1000 1020 1040 1060 1080 1100 1120 1140 1160 1180

FIG. 10g.

depends on the magnitude of the differences between the spectra of the two constituents, but is commonly of the order of 5–10%. This is adequate for many purposes. By more precise mathematical analysis of the curves much more accurate quantitative analyses can be made and are discussed in detail later (page 355). By the application of such qualitative and semiquantitative methods the number of chromatographic separations required for the complete analysis of the urinary steroids is greatly diminished.

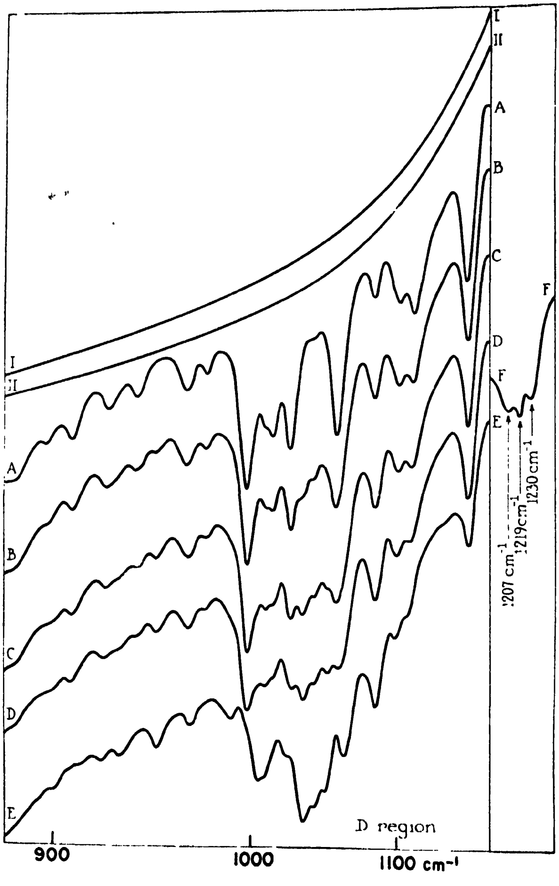
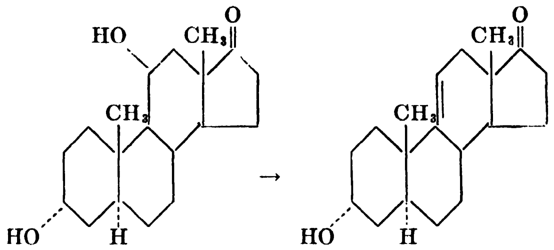


FIG. 11. Instrumental curves of androsterone (A) and etiocholanolone (E) and a series of their mixtures (B, C, D).

c. Detection of New Steroids. It will be apparent that in the course of such separation procedures, eluates will be obtained which exhibit spectra differing significantly from those of the known pure compounds and of their mixtures in the recognized sequence of elution. In such instances the presence of at least one new compound is indicated. Such a compound may be present only as a minor constituent of a mixture and its detection by chemical methods would be impossible.

The isolation of $\Delta^{9:11}$-androstenolone from urine provides an illustration of this application (Dobriner, 1948a). It was observed regularly that, *after* a series of eluates showing strong, typical androsterone absorption (Fig. 12), there followed a number of fractions which exhibited a slight but significant shift in the position of certain of the absorption bands. These are indicated in Fig. 12 by arrows. The presence of a new compound was accordingly inferred. Its separation from androsterone was not easy, and this indicated a close structural similarity, which was confirmed by the ultimate isolation of $\Delta^{9:11}$-androstenol-3 α-one-17 (XXXIV) the spectrum of which is shown in Fig. 12. Before sufficient of this material was available for chemical analysis, its probable structure was assumed and a sample was obtained. The infrared spectrum was found to be identical with that of the product isolated from urine.


XXIII XXXIV

The common physical properties of $\Delta^{9:11}$-androstenolone are closely similar to those of androsterone. $\Delta^{9:11}$-Androstenolone is invariably present in the α-ketosteroid fraction of hydrolyzed urine, so that the "androsterone" previously reported as isolated from urine by us and other investigators was actually a mixture of these two compounds.

This error led to a misunderstanding of the nature of the androsterone precursors. In certain adrenal disorders associated with adrenal hyperplasia of the virilism type the output of the so-called "androsterone" is greatly increased. Infrared studies have shown that in this condition androsterone itself is present only in normal amounts, but the excretion of $\Delta^{9:11}$-androstenolone is considerably enhanced. $\Delta^{9:11}$-Androstenolone is produced during hydrolysis of the urine by dehydration of androstenediol-3 α,11 β-one 17 (XXXIII) a metabolite of an adrenal precursor, characterized by the 11-oxygen function, while androsterone is presumably a metabolite of testosterone (XVII) (Dobriner *et al.*, 1948c).

A similar experience was encountered in regard to etiocholanolone.

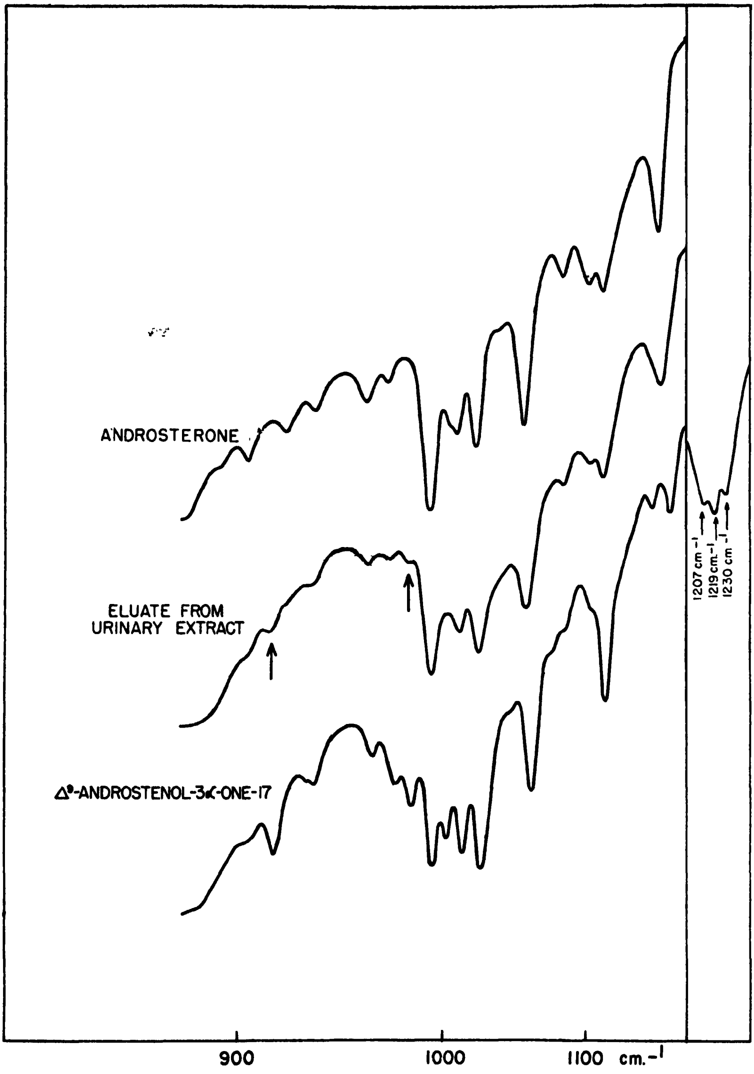


FIG. 12. Instrumental curves illustrating the detection and identification of Δ^9-androstenolone.

In certain instances the infrared spectra of the chromatographic fractions immediately *preceding* those containing pure etiocholanolone exhibited slight, but systematic differences. This observation led to the isolation of $\Delta^{9:11}$-etiocholenol-3-α-one-17 ($\Delta^{9:11}$-etiocholenolone), a dehydration product of etiocholanediol-3-α,11-β-one-17, (11-hydroxyetiocholanolone).

In distinction from the corresponding androstane derivative, discussed above, this compound has been seen only on rare instances in the urine of healthy individuals, but is present very frequently in the urine from patients with malignant growths (Dobriner *et al.*, 1947a, 1947b, 1948a).

A great variety of other specific absorption spectra have been recognized in a similar manner during the course of a systematic survey of the steroid content of urine under various conditions of health and disease. The correlations established between chemical structure and spectra of steroids, reviewed in the previous section, have aided greatly in the elucidation of the structure of many of these newly isolated compounds.

In addition, an extensive reference atlas has been prepared, containing the spectra of steroids, which, from biochemical considerations, might be expected to occur in urine and a number of steroids have been synthesized especially for this purpose. A number of steroids isolated from urine have been identified by comparison with such reference spectra. The case of $\Delta^{9:11}$-androstenolone has been discussed above in detail; other examples are listed in Table VI (Lieberman *et al.*, 1948b, 1949b).

TABLE VI

Steroids Isolated from Urine and Characterized by Infrared Spectrometry

Δ^1-Androstenedione-3,17
Δ^4-Androstenol-17-one-3 (*Testosterone*)
Androstanol-3 α-dione-11,17 (*11-Ketoandrosterone*)
Etiocholanol-3 β-one-17
Etiocholanediol-3 α,11-β-one-17
Pregnanol-3 α-dione-11,20 (*11-Ketopregnanolone*)

d. Steroids Labeled with Deuterium. The effect of introducing a deuterium atom on the infrared spectrum of a steroid has been described (page 311). By the application of infrared spectrometry the presence or absence of deuterium in the primary fractions (Chart IV) can be established, from measurements in the region of 2000–2200 cm.$^{-1}$ and from changes in the fingerprint region (Fig. 6). The presence of deuterium in the chromatographed fractions can be recognized in a similar way, and measurements of the absorption in the finger print region permits the individual deuteriated compounds to be identified.

The C—D absorption bands in the neighborhood of 2150 cm.$^{-1}$ are particularly useful for the identification of deuterium-containing steroids, since, at the concentrations employed, this region of the spectrum is transparent in the absence of C—D bonds in the molecule. The C—D absorption bands not only afford a positive indication of deuterium enrichment, but also provide a basis for the determination of the percentage excess of isotope in the sample. By this means the presence of deuterium has been established in 25 γ of a steroid containing five atoms per cent excess of the isotope (Dobriner *et al.*, 1949b).

Such deuteriated steroids are being employed in an endeavor to obtain a better understanding of normal and abnormal steroid metabolism. An illustration of the application of infrared spectrometry in such studies is provided by the following metabolic experiment. Allopregnan-5,6-d_2-ol-3 α-one-20-acetate was injected into a normal woman. After collecting the urine for 20 days, fractionation was effected by the procedures outlined in Chart IV. Feces collected during the experimental period were studied in analogous fashion. An examination of the region of the spectrum in which the C—D stretching bands occur demonstrated that the isotope was present in both the α- and β-hydroxyketone primary fractions, as well as in the α- and β-hydroxy nonketonic fractions from both urine and feces. No such bands were detected in the neutral nonketonic-nonalcoholic fractions. After chromatographic analysis of the deuterium-containing material, certain fractions exhibited strong C—D bands near 2150 cm.$^{-1}$ while other fractions lacked these bands or had bands of very low intensity. It was thus possible to locate rapidly the metabolites containing deuterium among the many substances excreted by a normal individual.

The sensitivity of infrared spectrometry for the analysis of deuterium in organic compounds is inferior to that of mass spectrometry, but metabolites of steroid hormones frequently occur in such small amounts that without some prior knowledge of the approximate isotopic content analysis by the mass spectrometer is difficult or impossible. Such preliminary information can be obtained rapidly by infrared spectrometry on microgram quantities of material without destruction of the compound.

2. *Quantitative Applications*

The techniques of quantitative infrared analysis are based on the same fundamental principles as those employed in visible and ultraviolet spectrophotometry.

In the discussion of the measurement of the absolute intensity of infrared absorption bands (page 330), it was pointed out that the determination of the molecular extinction coefficients of infrared absorption bands involves considerations which do not enter into the analogous problem in the visible and ultraviolet (page 331). For the purposes of quantitative analysis the calibration curves must be established and the analyses performed on the same instrument under identical conditions.

Figure 13 is a typical calibration curve for the quantitative analysis of a solution of dehydroisoandrosterone. It represents a plot of the optical density (log I_0/I) against the concentration for a selected absorption maximum. The slit width and cell thickness employed are standardized.

Such a curve may be used to determine the purity of a sample of a steroid. A more accurate assay is obtained if the measurements are made at two or more frequencies, selected at well separated positions in the spectrum. In this manner the purity of a steroid may be determined with an accuracy of about 1%.

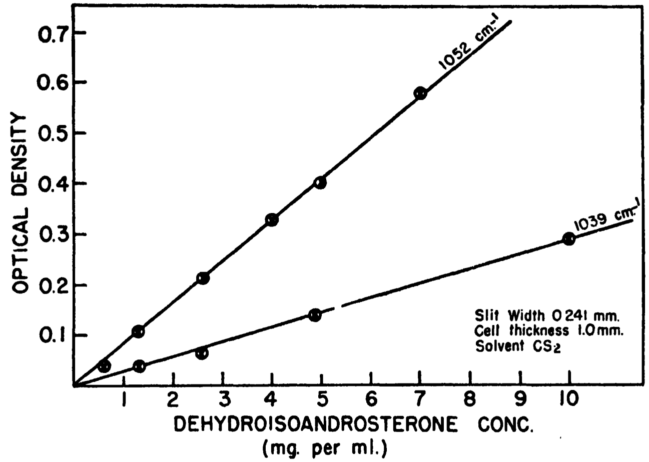


FIG. 13. Calibration curve for the quantitative estimation of dehydroisoandrosterone solutions.

Analysis of Mixtures. Although as yet quantitative infrared analysis has not been used extensively in the steroid field, the possible applications are manifold.

At present, two methods of quantitative infrared analysis are being applied to steroid analysis. These may be designated the "ratio method" and the "absolute method." The latter provides an absolute determination of the masses of each of the constituents present in the mixture, while the former, which is more rapid and simpler to apply, yields only the relative proportions of the constituents.

1. *The ratio method.* Several methods of quantitative analysis based on an empirical graphical treatment of the absorption curve have been described in the literature (Heigl *et al.*, 1947; Wright, 1941). The ratio method which has been utilized for the analysis of mixtures of isoandrosterone and dehydroisoandrosterone is independent of the cell thickness and of the total concentration of the solute; it depends only on the ratio of the concentration of the constituents and on the slit width employed.

The absorption spectra of dehydroisoandrosterone and of isoandrosterone over the range from 1025 to 1075 cm.$^{-1}$ are shown in Fig. 14. Analyses are based on measurements of the ratio of the absorption

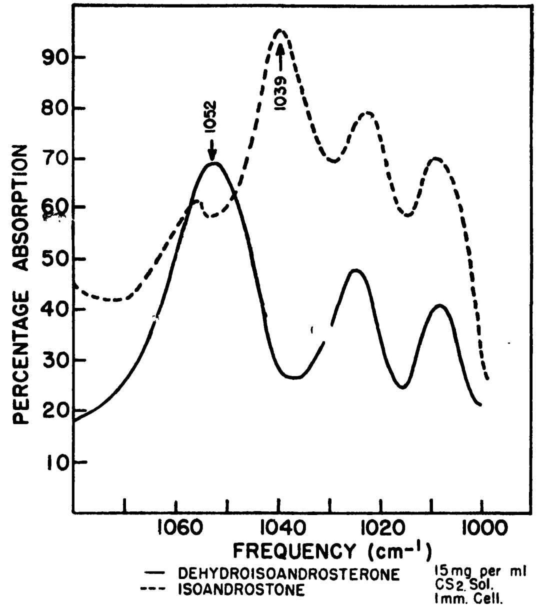


FIG. 14. Infrared spectra of dehydroisoandrosterone and isoandrosterone over the region utilized for the quantitative analysis of binary mixtures (carbon disulfide solution).

densities at the frequencies of the peaks (1052 and 1039 cm.$^{-1}$). In Fig. 15 the ratio

$$\frac{\left(\mathrm{Log}\,\frac{I_0}{I}\right)_{1039}}{\left(\mathrm{Log}\,\frac{I_0}{I}\right)_{1052}}$$

is plotted against the composition of a series of standard mixtures of the two steroids. The standard solutions were made up to contain approximately 6 mg. of total solute in 0.4 mm. of carbon disulfide, and a cell of approximately 1 mm. thickness was employed. The slit width was maintained exactly at 0.075 mm.

To analyze a mixture of unknown concentration, a solution is prepared which will give between 50 and 25% transmission at the peak frequencies, the slit width is set at the predetermined value and the ratio of the densities at 1039 and 1052 cm.$^{-1}$ evaluated. The concentration ratio may then be read off from the calibration chart.

The frequencies selected for measurement should be chosen so as to provide the maximum change in density ratio with change in composition. In the ideal case this will occur where a maximum in the spectrum of one constituent coincides with a minimum in the spectrum of the other. It is also preferable to select the maxima in the same narrow frequency range so that instrumental differences due to stray radiation and drift during the measurement are minimized. The steepness of the calibration curve is increased by diminishing the slit width.

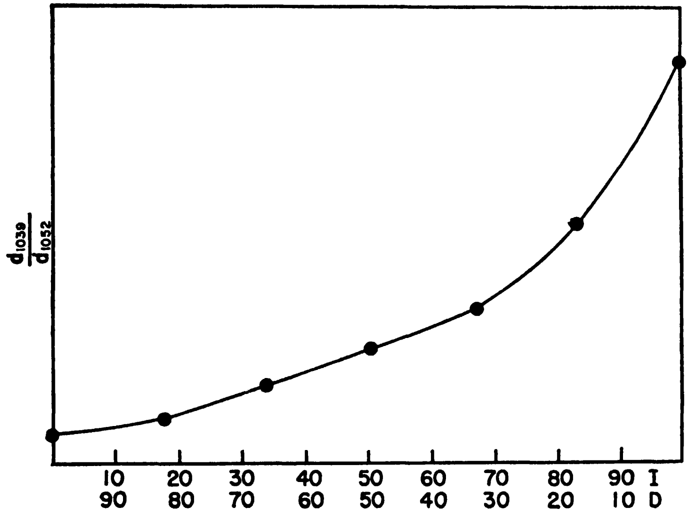


FIG. 15. Calibration curve for the quantitative analysis of mixtures of dehydroisoandrosterone (D) and isoandrosterone (I) by the ratio method.

2. *The absolute method* has been applied for the analysis of mixtures of pregnanediol-3-α,20-α diacetate and allopregnanediol-3-α,20-α diacetate, obtained in the course of chromatographic analysis of these difficultly separable steroids. Its application to the analysis of mixtures of estrone, equilin and equilenin (as the benzenesulfonyl esters) has been described by Carol *et al.* (1948) and reference should be made to their paper for experimental details.

3. *Application of Structure Correlations*

Information concerning the structure of steroids which can be derived from the location of the absorption bands between 3600 and 1200 cm.$^{-1}$ has been described in Section V. When applied to newly isolated compounds, these relations between structure and spectra have proved very helpful as they permit the possible structures to be reduced to a relatively small number. In certain cases the structure of a new compound has been assumed on the basis of the spectrum, taking into consideration also the behavior on chromatographic analysis and the biochemical feasibility. The substance with the assumed structure has then been synthesized, and the two spectra compared in the fingerprint region.

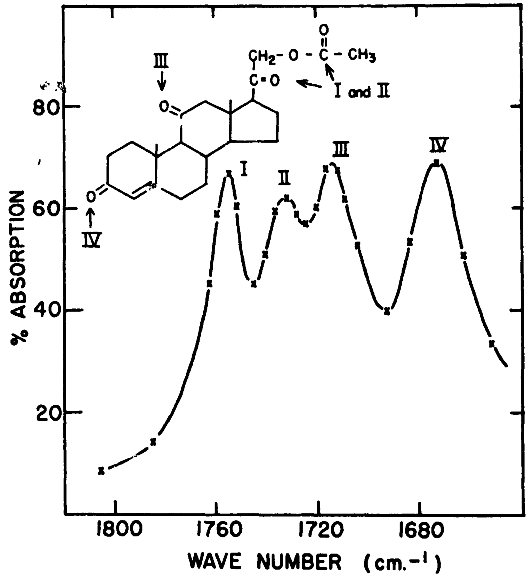
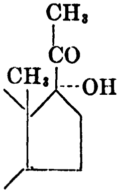


FIG. 16. Spectrum of dehydrocorticosterone acetate in the carbonyl stretching region (CaF_2 prism, carbon disulfide solution).

The number and location of the bands in the carbonyl stretching region is of particular value in this connection. The significance of this may be illustrated by the spectrum in Fig. 16 which shows the carbonyl stretching region of 11-dehydrocorticosterone acetate. Four bands are present, at 1758, 1732, 1716 and 1674 cm.$^{-1}$ From Charts I and II it can be deduced that the bands at 1758 and 1732 are associated with the 21-acetoxy-20-ketone group, and this is confirmed by the additional observation that an intense band is present also with maximum at 1228 cm.$^{-1}$ (see page 329). The maximum at 1674 cm.$^{-1}$ is typical of the Δ^4-3-ketone, and this leaves only the band at 1716 to account for. Taking into consideration the inferences made from the location of the other three bands, this remaining peak may be attributed to an 11-ketone, a 7-ketone or (less probably) a 12-ketone.

Of special interest in regard to steroid metabolism are compounds with an 11-oxygen function or with a tertiary hydroxyl group at position 17 (XXXV).



XXXV

Information which can be derived about such groups from infrared spectrometry is considerably extended by a knowledge of the chemistry of the compounds. For example, if a ketone absorption band is observed at 1716–1710 cm.$^{-1}$ in a "non-ketonic" fraction it may confidently be attributed to an 11-ketone group, since this group is known to resist coupling with the Girard reagent. Several 11-ketosteroid alcohols have been isolated and identified in this manner.

Similarly if after acetylation, the infrared spectrum still indicates the presence of a hydroxyl group, it can be inferred that an 11-β-hydroxyl group or a tertiary 17-hydroxyl group is present, since both of these fail to acetylate under the reaction conditions normally employed.

The measurement of the absolute intensities of the carbonyl bands yields additional information. Thus such intensity measurements permit distinction between a mono-, di- and triacetate (Table V). In the case of a compound which yields a single absorption band near 1740 cm.$^{-1}$ with an additional strong band at 1240 cm.$^{-1}$, a determination of the intensity of the 1740 cm.$^{-1}$ band and of the ratio of the intensities of the two bands will immediately indicate whether or not the compound contains a 17-ketone group in addition to the acetate group, and also how many acetate groups are present.

It is obvious that such techniques are not only applicable to compounds isolated from natural sources but also they can aid the investigator in synthetic organic chemistry in dealing with problems of molecular structure.

VII. CONCLUDING REMARKS

The subject of this article has been confined to the specific task of illustrating how infrared spectrometry has been utilized in the elucidation of the structure of steroids and in steroid metabolism. The specificity of the infrared spectrum is also finding application in connection with problems of steroid synthesis.

It has made possible the detection and identification of individual steroids and, in combination with adsorption chromatography, has permitted qualitative and quantitative analyses of complex mixtures of steroids to be performed. By the application of these methods, a considerable number of new steroids have been isolated from human urine. The introduction of deuterium atoms modifies the infrared spectra of steroids and infrared spectrometry is a valuable supplement to mass spectrometry in the detection, analysis and identification of deuterium enriched compounds.

Correlations between the positions of certain of the absorption bands and the molecular structure have been established. These permit the presence of all the functional groups which commonly occur in steroids to be recognized. In the case of carbonyl groups and double bonds more specific information can also be derived as to their location in the molecule. By the use of microtechniques it has been made possible to obtain this information on quantities of material of the order of 25 microgram.

Infrared spectrometry can be applied in a similar manner in connection with many other problems in biochemical and medical research. Its decisive part in the elucidation of the structure of the penicillins has been reviewed by Thompson *et al.* (1949), and it has been utilized for their quantitative analysis (Barnes *et al.*, 1947). Evidence is being accumulated by several investigators which indicates that infrared methods are applicable to the analysis and identification of nucleic acids, pyrimidines, amino acids, peptides, proteins, alkaloids, and fatty acids. There can be no doubt that it can be utilized also in the study of many other types of compounds.

In conclusion we wish to thank the several investigators who collaborated in supplying many of the compounds used in these studies. Individual acknowledgments have been made elsewhere (Jones *et al.*, 1948c, 1949). Our thanks are also due to Miss L. Groth, Mrs. P. Humphries, Mr. D. S. Keir, Mr. R. Lauzon, and Miss L. Packard for technical assistance. The work at the Sloan-Kettering Institute was supported by grants from the American Cancer Society (on recommendation of the Committee on Growth of the National Research Council), Ayerst, McKenna and Harrison, Ltd., the Jane Coffin Childs Memorial Fund for Medical Research, the Commonwealth Fund, the Anna Fuller Fund, the Lillia Babbit Hyde Foundation, International Cellucotton Products Company, the Albert and Mary Lasker Foundation, the Adele R. Levy Fund, the National Cancer Institute of the National Institute of Health, U. S. Public Health Service, the New York Foundation, and the Sidney Rheinstein Fund.

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Alloxan Diabetes

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I. INTRODUCTION

1. *History*

Alloxan, the ureide of mesoxalic acid, produces a selective necrosis of beta cells in the islets of Langerhans of the pancreas when injected into animals. This is followed in 24 to 48 hours by the development of diabetes mellitus.

The early history of alloxan is meager. Although alloxan was synthesized over a century ago (Woehler and Liebig, 1838) there was very little interest in this substance until recently. Liebig (1862) reported that he found alloxan in the intestinal mucus of a patient and later Lang (1866) detected alloxan or its precursor in human urine. The excretion of alloxan was studied by Cerecedo (1931).

Jacobs first reported that when alloxan is injected intravenously into rabbits a transitory hyperglycemia develops which is followed by profound hypoglycemia with convulsions and death in 7 to 10 hours unless glucose is given (Jacobs, 1937). He made no histological examination and was unable to explain the hypoglycemic action of alloxan.

Six years later Dunn *et al.* (1943), apparently unaware of Jacobs' work, injected alloxan among other chemicals intravenously into rabbits, in attempting to explain the pathogenesis of the crush syndrome. When alloxan was injected their animals died during the first day or so with "distinctive symptoms which were not related to renal disease." The pancreases of their rabbits on histological study revealed a selective necrosis of the islets of Langerhans.

Brunschwig *et al.* (1943) next reported that they had observed rabbits "which survived injections of alloxan and exhibited only transitory hyperglycemia." In five dogs they reported "hyperglycemia sustained for two to three weeks." Permanent diabetes produced with alloxan in rabbits was reported by Bailey and Bailey (1943), in rats by Dunn and McLetchie (1943) and in dogs by Goldner and Gomori (1943).

2. Animals Susceptible to Alloxan

Since then it has been established that many animals are susceptible to the diabetogenic action of alloxan. Table I lists these animals and indicates an appropriate diabetogenic dose and its recommended method of administration.

The guinea pig appears resistant to the diabetogenic action of alloxan, and according to Mirsky (1945) alloxan destroys the islets of Langerhans in the duck but fails to produce diabetes. Owls and chickens also fail to develop diabetes when given alloxan (Scott *et al.*, 1945). Although the frog develops islet lesions, hyperglycemia is not produced (Seiden, 1945). The toad develops neither hyperglycemia nor pancreatic changes following alloxan (Houssay *et al.*, 1945b).

The dose of alloxan required to produce diabetes varies with the animal. If too small an amount is given diabetes is not produced, whereas if too large a dose is employed serious kidney damage with uremia may result. This point is well illustrated by the experiments of Goldner and Gomori (1943) with dogs. When doses of alloxan of more than 100 mg./kg. were used in a single intravenous injection it was fatal within a few hours. A dose of 75 to 100 mg /kg. produced a hyperglycemic-uremic syndrome in which the dogs succumbed to uremia within 4 to 7 days. With a dose of 50 to 75 mg. of alloxan per kilogram typical diabetes mellitus with characteristic polydipsia, polyuria, hyperglycemia, and glycosuria was produced. Finally a single dose of 25 mg./kg. was ineffective.

The preferable mode of administration of alloxan varies with the animal used. The intravenous method is usually preferred for the larger animals while the subcutaneous route is more feasible for small animals such as the rat. In the rat the intravenous diabetogenic dose is 40 mg./kg. (Lazarow and Palay, 1946) whereas the subcutaneous dose is larger, 150–200 mg./kg. of body weight.

TABLE I

Diabetogenic Dose of Alloxan

Animal	Dose in mg./kg.	Reference
1. Dog	50–75 i.v.	Goldner and Gomori, 1943
2. Rabbit	150–200 i.v.	Bailey and Bailey, 1943
3. Rat	150–200 s.c. 40–50 iv.	Dunn and McLetchie, 1943 Lazarow and Palay, 1946
4. Mouse	75 i.v.	Lazarow, 1947
5. Cat	500–1000 orally	Ruben and Yardumian, 1946
6. Monkey	150–300 i.v.	Banerjee, 1944
7. Sheep	90–200 i.v.	Jarrett, 1946
8. Goat	100 i.v.	Saviano and De Franciscis, 1946
9. Hamster	60 i.v.	Harris *et al.*, 1946
10. Pigeon	125–200 i.v.	Goldner and Gomori, 1945
11. Turtle	400 i. pul.	Garcia Ramos, 1945
12. Dogfish	120–150 i.p.	Saviano and De Franciscis, 1946
13. Ape	600 i.v.	Siliprandi, 1947

KEY
i.v = intravenous injection
s.c. = subcutaneous injection
i.p. = intraperitoneal injection
i. pul. = intrapulmonary injection

In the cat diabetes can be produced by giving very large doses orally, mixed with the food, while in the turtle an alloxan solution may be injected into the lung.

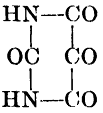
Whereas alloxan is effective intraperitoneally in the rat the degree of liver damage tends to be greater, hence the subcutaneous or intravenous route of administration is to be preferred.

The speed with which the alloxan is injected is important, for a diabetogenic dose given intravenously too slowly may not produce diabetes (Houssay *et al.*, 1946). Ordinarily the administration intravenously of the entire dose within 2 to 3 minutes is satisfactory. Presumably when the injection is made too slowly the alloxan is inactivated or neutralized before an effective concentration can be attained in the pancreas.

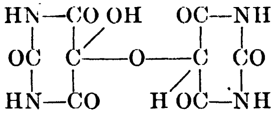
II. CHEMISTRY

1. *Chemical Properties of Alloxan*

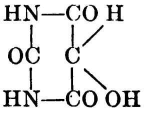
Alloxan is a pyrimidine, the ureide of mesoxalic acid. It can be obtained from uric acid by oxidation with nitric acid or it can be prepared from barbituric acid. It was first obtained from uric acid by Woehler and Liebig in 1838. It is a white powder easily soluble in water or alcohol. In solution it is distinctly acid and cannot be neutralized without inactivation. If alloxan is neutralized or is dissolved in either rabbit or human blood plasma its diabetogenic property is lost (Leech and Bailey, 1945).



Alloxan



Alloxantin



Dialuric acid

Alloxan may act either as a strong oxidizing agent or as a reducing agent. Acting as an oxidizing agent it is itself reduced to dialuric acid whereas acting as a reducing agent it is oxidized to parabanic acid. *In vitro,* alloxan was found to inhibit the conversion of glucose-1-phosphate (Cori ester) to glucose-6-phosphate (Robison ester). This is the second step in the accepted metabolism of glycogen. The inhibition was believed to be due to the action of alloxan as an enzyme destroyer (Lehman, 1939).

2. *Related Diabetogenic Substances*

The specificity of alloxan is quite striking, for if its chemical structure is appreciably changed its diabetogenic action is lost.

One molecule of alloxan may join with one molecule of dialuric acid, a reduction product of alloxan, to form alloxantin, and it has been shown that alloxantin produces diabetes when injected into rabbits (Koref *et al.,* 1944). A solution of alloxantin, however, changes into alloxan on standing and this change may occur in the blood stream after injection.

Dialuric acid, the reduction product of alloxan, is also diabetogenic (Bruckmann and Wertheimer, 1945). Besides alloxan, alloxantin, and dialuric acid, the following substances have been reported to be diabetogenic in animals: monomethylalloxan, monoethylalloxan, monopropylalloxan, dimethylalloxantin, diethylalloxantin and monomethyldialuric acid.

Violuric acid and barbituric acid are claimed by Gitter and Cardeza (1946) to be diabetogenic but others have thus far obtained negative results. One observer (Stoll, 1946) states that ninhydrin produces the same effects on the islets of Langerhans in rats and guinea pigs as alloxan but others have failed to find ninhydrin diabetogenic.

The use of alloxantin or dialuric acid as diabetogenic agents is less satisfactory than alloxan because of their relative insolubility.

It appears, therefore, that very little change can be made in the structure of alloxan if its diabetogenic action is to be preserved. As stated by Bruckmann and Wertheimer (Bruckmann and Wertheimer, 1947) an intact pyrimidine ring seems to be essential for diabetogenic action. Activity is abolished by substitution in the alloxan molecule at any position other than in one imino group.

III. Blood Alloxan

Alloxan can be detected in the blood and in the pancreas immediately after its intravenous injection into rabbits but has all been destroyed or removed from the blood within 5 minutes after its injection (Leech and Bailey, 1945).

1. Chemical Tests for Alloxan

Leech and Bailey (1945) described a method for the quantitative detection of alloxan in blood in quantities as small as 0.05 mg. based on the ability of alloxan to reduce potassium ferricyanide in the cold. A qualitative test is also described which depends on the ability of alloxan to reduce tungstic acid at room temperature.

A microbiological and fluorometric test to detect minute amounts of alloxan was described by Banerjee *et al.* (1945). This method employs the condensation of alloxan with 1,2 dimethyl-4-amino-5 (*d*-1′-ribitylamino)-benzene to yield riboflavin. The riboflavin in turn is measured by its fluorescence using a Coleman photofluorometer, model 12, or by its growth-promoting properties for *Lactobacillus casei.* Amounts of alloxan as small as 0.2 to 4 γ/cc. can be measured by the fluorescence properties, while amounts from 0.05 to 0.4 γ of alloxan could be measured by the microbiological procedure. These tests were made on solutions of pure alloxan.

Archibald (1945) described gasometric, titrimetric, three colorimetric and one fluorometric methods for the determination of alloxan. The fluorometric method, the most sensitive of those described, measured a concentration of alloxan of 0.02 mg. % (0.2 γ/cc.).

Karrer *et al.* (1945) describe still another method which detects alloxan in aqueous solution in concentrations as low as 0.5 γ/cc. This test is based on the measurement of the blue fluorescence in ultraviolet light of alloxazines produced by the reaction of alloxan and *o*-phenylenediamine.

Using their pyrrole test and "purple" test Tipson and Ruben (1945) claim to have detected the presence of a substance suggesting alloxan in many tissues of animals and humans. The liver had especially large amounts of this substance. Since these tests are not specific for alloxan, however, further investigation must be done before the presence of alloxan in tissues can be accepted.

The fate of alloxan in the body was discussed by Cerecedo (1931) who, on the basis of his and other work, believed that alloxan is to a large extent destroyed in the body. His experiments led him to believe that alloxan combines in the body with sulfuric acid and is possibly excreted in the bile as ethereal sulfate. A small quantity of alloxan is converted into alloxantin and excreted in the urine as murexide.

IV. ALLOXAN AND SULFHYDRYL COMPOUNDS

1. Blood Glutathione Changes

In an effort to elucidate the action of alloxan attempts have been made to detect some of its interactions with substances in the blood. In 1930 it was suggested by Labes and Freisburger that alloxan might react with sulfhydryl groups.

Leech and Bailey (1945) in studying the effect of alloxan upon blood sugar discovered that the nonglucose reducing substances in blood as well as the blood sugar were markedly decreased after the injection of alloxan into rabbits. As glutathione, a sulfhydryl compound, comprises most of the nonglucose reducing substances in blood, direct determinations of reduced glutathione were made using the Benedict-Gottschall method. They were able to show that an immediate and marked decrease of blood glutathione followed the injection of 200 mg./kg. of alloxan into rabbits, suggesting an interaction of these two substances. The concentration of thioneine, another sulfhydryl compound, was also decreased in blood following the injection of alloxan.

In three rabbits the blood glutathione reached zero immediately following alloxan, but reappeared in 1 to 2 minutes. In three other rabbits a 48, 57, and 61% reduction of the control value was obtained respectively at the end of the alloxan injection. The control value of glutathione was not reached again for 18 to 24 hours after injection, at which time diabetes began to appear.

Diabetes in rabbits can be produced by the repeated daily injection of subliminal doses (40 mg./kg.) of alloxan intravenously, as shown by Bailey, C. C. *et al.* (1944), who found that morning blood sugars gradually rose over a period of several days until diabetic levels were obtained. In such cases the reduced glutathione in the blood tended also to rise gradually until diabetes developed, at which time it suddenly fell to the preinjection level. These findings suggest the thesis that glutathione acts as a protective agent for the body against alloxan.

2. Prevention of Alloxan Diabetes

Since alloxan interacts with blood glutathione the possibility was considered that the latter may represent one of the defenses of the body against alloxan. Although Leech and Bailey (1945) were unable to prevent the diabetogenic action of alloxan by injecting the molecular equivalent of glutathione immediately before and during the injection of alloxan, Lazarow, by using much larger doses, succeeded (Lazarow, 1946). Lazarow was able to prevent diabetes in rabbits by giving 2.5 g./kilo of glutathione within 5 minutes before the injection of alloxan. He further found that large doses of cysteine (912 mg./kg.), thioglycolic acid or British Anti-Lewisite (BAL), all sulfhydryl compounds, had a similar protective action against alloxan (Lazarow, 1947a).

Three substances believed to react chemically with alloxan have been shown by Weinglass *et al.* (1945) to prevent the diabetogenic action of alloxan if given just prior to its injection. These are 3-4-diaminotoluene, orthophenylenediamine, and sodium bisulfite. Histological sections failed to show islet cell damage in the pancreas when protection was afforded by these chemicals.

Banerjee reports that nicotinic acid (100 mg./kg.) intravenously in five rabbits or intraperitoneally in six rats prevented diabetes if given at the same time as alloxan (100 mg./kg.). Both were given daily for 2 to 3 days (Banerjee, 1947). This dose of alloxan, however, is not always diabetogenic. When a larger dose of 200 mg./kg. each of nicotinic acid and alloxan was employed, two rabbits developed diabetes. Pyridine-dicarboxylic acid and 2-phenylquinoline-4-carboxylic acid which, like nicotinic acid, contain both the carboxyl group and the pyridine ring also prevents diabetes if given immediately before the alloxan is injected.

Banerjee also found that diabetes failed to develop when 1,2-dimethyl-4-amino-5(*d*-1′-ribitylamino) benzene was given to rabbits for 2 days at the same time alloxan, 100 mg./kg., was given. This chemical reacts with alloxan to form riboflavin (see section III, above).

It should be stressed that all the substances mentioned above which are claimed to prevent diabetes must be injected almost simultaneously with the alloxan; otherwise no protective action results. This would suggest that a chemical interaction may occur between the substance and alloxan, and inactivate the latter before the alloxan can reach the islets of Langerhans in the pancreas.

The injection of adrenalin intraperitoneally before the injection of alloxan prevents diabetes, as shown by Kass and Waisbren (1945). Bailey and LeCompte (1947) found that the injection of small quantities of epinephrine intraperitoneally into rats produces a marked constriction of the capillaries and small blood vessels of the intact pancreas which is apparent microscopically. It is presumably the vasoconstriction which prevents the alloxan from reaching the islets of Langerhans.

Walpole and Innis (1946) ligated the pancreatic ducts for 30–60 days, which resulted in pancreatic fibrosis but spared the islet tissue. When alloxan was injected into rabbits so prepared, diabetes did not ensue. Goldner and Gomori (1947) using dogs, however, have not been able to duplicate these results.

It has been claimed (Jimenez Diaz *et al.*, 1946) that temporary ligation of the renal arteries in dogs at the time alloxan is given intravenously prevents the development of diabetes. Both the dogs with clamped renal pedicle and control dogs showed the initial hyperglycemia and hypoglycemic phases but only the control animals developed diabetes. It has been suggested that clamping the kidney vessels might produce a reflex construction of the pancreatic vessels, although direct observations of the pancreas during the experiment failed to show any apparent change in the blood supply to the gland. Others (Martinez *et al.*, 1947) have found that in rats renal ischemia produced by renal artery ligation does not prevent the development of diabetes when alloxan is given.

TABLE II

Drugs Claimed to Prevent Action of Alloxan
Glutathione
Cysteine
Thioglycolic acid
B.A.L. (British Anti-Lewisite)
3,4-Diaminotoluene
Orthophenylenediamine
Sodium bisulfite
Adrenalin
Nicotinic acid
Pyridine-dicarboxylic acid
2-Phenyl-quinoline-4-carboxylic acid
1,2-Dimethyl-4-amino-5 (*d*-1′-ribityl-amino)-benzene

V. MODE OF ACTION OF ALLOXAN

1. *Relation to Endocrine Glands*

The exact way in which alloxan produces islet cell necrosis and diabetes is not known. The diabetes is believed to be the result of pancreatic islet destruction. It has been suggested that the islet cell destruction results from the action of alloxan upon cellular enzymes—possible sulfhydryl enzymes which are known to interact with alloxan (Lazarow, 1946).

Bruckmann and Wertheimer (1947) suggest that alloxan may act in one of the following three ways:

1. A selective accumulation of alloxan in toxic amounts in the beta cells. Since the beta cells comprise less than 0.5% of the pancreas this would still be possible despite the fact that they found the concentration of alloxan after injection lower in the pancreas than in liver or kidney.
2. Alloxan may compete with a structurally similar compound for an enzyme with resulting disorganization of the cell metabolism and eventual destruction of the cell. Thus far no data to substantiate this theory are available.
3. There may be a specific reaction of alloxan in the islet cell, with or without a selective accumulation. Such a reaction would probably consist in the inactivation of an enzyme system, which may be intimately concerned with insulin synthesis.

That the islet cell destruction is due to the direct action of alloxan was shown by Gomori and Goldner (1945), who temporarily ligated the arteries to one-half of the dog's pancreas, injected alloxan intravenously and 5 minutes later released the temporary arterial ligatures. On histological examination they found that in that part of the pancreas deprived of blood for the first 5 minutes after the alloxan was given the islets were normal whereas the other half of the pancreas which had not been deprived of blood showed islet cell destruction.

Recently Jimenez Diaz has reported some interesting but confusing data (Jimenez Diaz *et al.*, 1946). He claims that if alloxan is injected directly into the pancreatic artery diabetes does not develop. The intrasplenic injection of alloxan in a rat, however, produces diabetes (Janes, 1948).

Since the injection of pituitary extract is known to produce diabetes in dogs the possibility that alloxan may exert its diabetogenic effect through the pituitary was studied. It has been shown that diabetes develops when alloxan is injected into hypophysectomized rats, and histological sections reveal islet cell destruction (Gaarenstroom *et al.*, 1946; Bailey *et al.*, 1947). This offers strong evidence that alloxan does not act through the pituitary.

The effect of the thyroid gland on alloxan diabetes has been studied by Houssay and his associates (Houssay *et al.*, 1946). They report that thyroidectomized rats show a greater resistance to the diabetogenic action of alloxan than control animals and that thiouracil treated rats show an even greater resistance to alloxan. On the other hand experimental hyperthyroidism produced by the administration of thyroid extract temporarily increases the sensitivity of rats to alloxan.

The diabetogenic action of alloxan in dogs is not prevented by the removal of the adrenal medulla, thyroid or parathyroid glands (Houssay, 1947).

The effect of diet prior to the injection of alloxan has been studied by Martinez (1945). A diet rich in lard greatly increases the rat's sensitivity to alloxan. This diet loses its effect if methionine or thiouracil is added. A high protein diet, on the other hand, decreases sensitivity to alloxan. Kass and Waisbren (1945) have shown that the withdrawal of all food in adult rats for a period of 48 to 60 hours renders the rat more susceptible to alloxan.

2. Blood Sugar Changes Following Alloxan

Following the injection of alloxan most animals exhibit a transitory hyperglycemia followed in a few hours by hypoglycemia. The tendency to hypoglycemia in turn gives rise in 24 to 48 hours to final hyperglycemia or diabetes. The intensity of the hypoglycemic phase varies in different animals. In the rabbit hypoglycemic convulsions with death may occur if the hypoglycemia is untreated, especially if the animal is fasted during the experiment. In the dog and rat, however, hypoglycemic convulsions are infrequent and less precaution is needed.

Many experiments have been carried out to explain these blood sugar variations. Goldner and Gomori (1944b) believe that the initial hyperglycemia is the result of adrenalin stimulation of gluconeogenesis while the hypoglycemic phase is due to released insulin from destroyed islets of Langerhans. Another interpretation is offered by Houssay *et al.* (1945a) who attribute the initial hyperglycemia to a direct action of alloxan on the liver and the hypoglycemic stage to an extrapancreatic effect, probably to a lack of glucose production by the liver.

Neither the initial hyperglycemia nor the subsequent hypoglycemia is necessary for the destruction of the islets of Langerhans or the development of diabetes, for the initial hyperglycemia can be prevented by insulin and the hypoglycemia by glucose injections, yet the beta cells degenerate and diabetes develops (Goldner and Gomori, 1944a). Different theories regarding the mechanism of the blood sugar changes following injection of alloxan have been summarized recently by Lukens (1948) in an excellent review article.

VI. PATHOLOGY

The pathology of alloxan diabetes has been reviewed in detail by Duff (1945) to which the reader is referred for descriptions of the effect of alloxan upon various tissues.

When usual diabetogenic doses of alloxan are employed, significant and permanent pathological changes are essentially limited to the islets of Langerhans in the pancreas.

Histologic changes in the islet cells are discernible 5 minutes after the injection of alloxan into rats (Hughes *et al.*, 1944) and rabbits (Bailey, C. C. *et al.*, 1944). To study the development of pancreatic islet lesions Bailey, O. T. *et al.* (1944) anesthetized rabbits with nembutal and obtained serial biopsies from the pancreas at varying intervals after the administration of alloxan in dosage of 200 mg./kg.

In the specimen obtained at 5 minutes after the injection of alloxan some loss of granules from the cytoplasm of islet cells was seen and the nuclear membranes of many islet cells, especially the beta cells, were more prominent.

Biopsies made at 10 and 15 minutes after the injection of alloxan showed that most of the granules had disappeared from the cytoplasm of the cells in the centers of the islets. The peripheral alpha cells appeared normal. At 30 and 45 minutes there was further loss of granules from the central beta cells and their nuclei were more shrunken but the chromatin was still granular. The alpha cells remained unchanged. At 1 hour and 1½ hours after alloxan the central cells were further shrunken and the chromatin formed a compact deeply staining mass. Early cytoplasmic disintegration was seen in some areas.

At 2 hours distortion of the general architecture of the islets was apparent. Three hours after alloxan the nuclei of most of the islet cells were pyknotic and the cytoplasm was irregular in outline. In 4 hours the cells with degenerating cytoplasm had coalesced somewhat so that individual islets appeared smaller. At 5 and at 6 hours biopsy specimens revealed further coalescence of the islet cells and complete disintegration of some cells.

At no time was polymorphic neutrophil infiltration seen and the blood vessel walls, stroma, and pancreatic parenchyma remained unchanged.

Three days after alloxan was given all of the degenerating cells in the islets had disappeared. The center of the islets contained normal stroma cells with a collar of normal peripheral alpha cells. The islets appeared somewhat collapsed and flattened.

Rabbits sacrificed 2 months after the injection of alloxan with the production of alloxan diabetes revealed islets that were smaller than normal, but otherwise appeared normal with the usual stains. One rabbit pancreas stained by Gomori's method revealed islets consisting entirely of alpha cells; no beta cells could be identified. Since the islets seemed less numerous than in control biopsies it is probable that some islets had disappeared entirely.

Thus it would appear that alloxan causes destruction of the beta cells of the islets of Langerhans with persistence of small numbers of alpha and nongranular cells without significant injury to the acinar tissue of the pancreas.

Diabetes can be produced in rabbits by the repeated daily injection of small subliminal doses intravenously. When alloxan in a dosage of 40 mg./kg. is given daily the pathology in the pancreatic islets of Langerhans differs somewhat from the picture described above. Under these conditions hydropic degeneration of the beta cells is seen, although not as extensively as that observed following the production of diabetes with anterior pituitary extract in dogs. In addition to hydropic degeneration, a few mitotic figures and degranulation of the beta cells are found if the rabbit is sacrificed within a day or two following the onset of diabetes (Bailey, O. T. *et al.*, 1944). Hydropic degeneration of the beta cells has also been observed in rabbits two months after the development of alloxan diabetes (Kennedy and Lukens, 1944). Presumably these cells represent the effect of hyperglycemia upon beta cells that escaped destruction with alloxan.

Thus far no one has reported in alloxan diabetes, fibrosis or hyalinization of the islets, the most frequent lesions found in human diabetes.

With the usual diabetogenic dose the lesions found in the kidney, which are usually limited to the renal tubule epithelium, are reversible. Changes in the liver cells especially fatty infiltration and small areas of focal necrosis do not appear to represent permanent damage. Minor cytological changes have been reported in the adrenals and pituitary.

Complications:

Some of the complications of diabetes observed in humans have also been reproduced in the alloxan diabetic animal.

Acidosis and coma (Bailey, C. C. *et al.* 1944; Kaplan *et al.*, 1945) have been reported in rabbits and rats. In rabbits, marked hyperlipemia with lipemia retinitis has been observed. The tendency to acidosis in alloxan diabetic dogs is less than in depancreatized dogs even though the diabetes in the former may be more severe (Thorogood and Zimmerman, 1945).

Diabetic cataracts develop rather rapidly in rabbits and rats with alloxan diabetes, especially if their blood sugar is allowed to remain high (Bailey, C. C. *et al.* 1944). If the diabetes is severe cataracts may appear within 4 to 6 weeks but treatment with insulin seems to delay their appearance. Retinal hemorrhages have been reported by only one group of observers who state that capillary hemorrhages were detected both by an ophthalmologist and on histological examination (Lewis *et al.*,1947).

It is of considerable interest that neither diabetic neuritis nor arteriosclerosis has been observed in alloxan diabetic animals.

VII. COMPARISON WITH OTHER DIABETOGENIC METHODS

Permanent diabetes can be produced in animals by the repeated injection of crude anterior pituitary extract (APE), pancreatectomy, the repeated injection of glucose or a single injection of alloxan (or closely allied compounds as alloxantin or dialuric acid which possibly are converted to alloxan).

1. Pituitary Diabetes

Some of the striking differences between diabetes produced by alloxan and APE have been pointed out by Bailey *et al.* (1947).

(1) Diabetes may be produced by APE only in the dog and in the partially depancreatized rat or cat, whereas alloxan is effective in the dog, rat, cat, rabbit, ape, monkey, goat, sheep, mouse, hamster, pigeon, turtle and dogfish.

(2) Hyperglycemia is imperative for the production of diabetes with APE. If hyperglycemia is prevented by the use of insulin, phlorizin or a low carbohydrate diet diabetes does not develop. Alloxan produces diabetes irrespective of hyperglycemia.

(3) Alloxan produces islet cell destruction and diabetes within 24 hours whereas APE must be reinjected daily for several days or weeks.

(4) The pancreatic islet lesions produced by a single injection of alloxan show degenerative changes from the start with a destruction of all or nearly all of the beta cells within 24 hours. The islet cell changes with APE develop much more gradually, with degranulation and extensive hydropic degeneration of the beta cells. Although hydropic degeneration may be seen when diabetes is produced by the repeated injections of subliminal doses of alloxan it is not nearly so extensive as with APE.

2. Pancreatectomy

As compared with pancreatectomy the production of diabetes with alloxan is far simpler both in time required and necessary technique. In some animals such as the rabbit the pancreas is quite diffuse and for this reason a complete pancreatectomy is quite difficult and at times not possible. In order to produce diabetes with certainty at least nine-tenths of the pancreas must be removed, as shown by the original investigation by Minkowski (1893).

The histologic findings in the pancreatic remnant after partial pancreatectomy include loss of granules of the beta cells in the islets of Langerhans followed by hydropic degeneration (Allen, 1922). These findings simulate the changes observed after the administration of crude anterior pituitary extract but are quite different from the usual findings in alloxan diabetes previously described.

The administration of alloxan to previously pancreatectomized rats produces no significant change in the blood sugar (Foglia *et al.*, 1944).

With pancreatectomy the external pancreatic secretions are lost whereas in alloxan diabetes acinar function is preserved.

When dogs made diabetic with alloxan are then depancreatized the insulin requirement decreases markedly (Thorogood and Zimmerman, 1945) often to one-third its previous amount. Pancreatic duct ligation, however, caused little or no decrease in the insulin requirement in alloxan diabetic dogs. As mentioned earlier the tendency to acidosis is less in alloxan diabetic dogs than in depancreatized dogs even though the diabetes in the former may be more severe (Thorogood and Zimmerman, 1945).

3. Glucose Diabetes

If hyperglycemia is maintained in cats by the repeated intraperitoneal injection of glucose hydropic degeneration of the beta cells in the islets of Langerhans and finally permanent diabetes is produced (Dohan and Lukens, 1947). In general, alloxan diabetes differs from diabetes produced with glucose in the same way that it differs from pituitary diabetes, namely; (1) Diabetes has been produced with glucose only in the cat, in contrast to the many animals subject to alloxan diabetes. (2) Hyperglycemia is imperative for diabetes produced with glucose but not for diabetes produced with alloxan. (3) Alloxan produces islet cell destruction and diabetes within 24 hours whereas diabetes produced by glucose requires many injections over several days. (4) The pancreatic islet lesions produced with alloxan show degenerative changes from the start whereas with diabetes produced by glucose the islet cell changes show extensive hydropic degeneration which simulate the islet changes following the injection of pituitary extract.

The hyperglycemia produced with glucose in its initial development, however, differs from that produced with pituitary extract in several important respects, as pointed out by Dohan and Lukens (1947). "In the first 3 to 4 days, before lesions of the islands appear, crude anterior pituitary extract lowers the R.Q., inhibits the action of insulin on the blood sugar, promotes ketogenesis and thyroid and adrenal hyperplasia occur. There is probably a direct inhibition of the secretion of insulin and the insulin content of the pancreas is reduced. In contrast, the administration of glucose initially raises the R.Q., increases the efficiency of injected insulin, is antiketogenic and is not accompanied by thyroid or adrenal hyperplasia. Furthermore, glucose stimulates insulin secretion and carbohydrate diet maintains a high insulin content of the pancreas."

Diabetes produced with the injection of glucose as with APE is dependent on the maintenance of hyperglycemia with its increased functional demands upon the islets of Langerhans and the subsequent appearance of pathological changes in the islets.

VIII. Effect of Alloxan upon Humans

Since alloxan destroys the insulin-producing beta cells in the islets of Langerhans in animals its use as a chemotherapeutic agent in humans with islet cell adenomas and clinical hyperinsulinism suggested itself. Brunschwig *et al.* (1944) administered alloxan to six patients with metastatic carcinoma, one with a malignant islet cell carcinoma and clinical hyperinsulinism.

In three patients treated with alloxan little or no effect upon the blood sugar could be detected. A fourth patient developed a severe reaction with a chill, nausea and cyanosis but no blood sugar change occurred. Another patient, who received the largest amount of alloxan (600 mg./kg.) developed hypoglycemia, the blood sugar having fallen to 16 mg. % 3 hours after injection. This was relieved with intravenous dextrose but death occurred 3 hours later. Postmortem studies revealed diffuse degenerative changes in the hepatic cells. The pancreas, however, revealed only questionable evidence of damage to a number of cells in some of the islets; many islets were unaffected.

The sixth patient had an islet cell carcinoma with hyperinsulinism. He received several series of daily alloxan injections and after each a period of freedom from hypoglycemic attacks for 10 to 21 days was observed. At postmortem examination no evidence of damage to either normal islet cells of the pancreas or to the metastatic nodules in the liver could be seen.

Relief of hypoglycemic symptoms was reported in an 8-month-old baby girl with clinical hyperinsulinism by the use of alloxan after exploratory laparotomy had failed to reveal a pancreatic adenoma (Talbot *et al.*, 1948). In this child two series of injections were given. The first series began with very small doses (20 mg./kg.) which were gradually increased daily to 100 mg./kg. over a period of 7 days.

At the end of these injections, when a total of 3.3 g. of alloxan had been given, the fasting blood sugar values had returned to normal and the tendency to hypoglycemia had disappeared. Fourteen days later the fasting blood sugar began to fall. Three weeks after the first course of alloxan a second series of injections was begun, giving 100 mg./kg. of alloxan for 7 days and 200 mg./kg. on the eighth day. Again evidences of hypoglycemia disappeared and diabetes did not develop. Although she did not again develop evidence of hypoglycemia, later studies revealed internal hydrocephalus which may have been the result of brain damage from repeated severe hypoglycemia.

Flinn *et al.* (1947) could detect no clinical effects from the administration of alloxan to a patient with islet cell carcinoma of the pancreas with liver metastases.

Conn showed that alloxan may destroy the normal islets of Langerhans in the pancreas (Conn *et al.*, 1947). He treated a patient with hyperinsulinism with alloxan and although some improvement occurred, the patient continued to have morning hypoglycemic attacks. The patient finally submitted to surgery and an islet cell tumor was removed which histologically failed to reveal any evidence of necrosis. A biopsy of the pancreas, however, showed that the normal islet cells had been damaged. The patient was diabetic following surgery presumably from damage to the islets of Langerhans by the previously administered alloxan.

The great danger associated with the administration of alloxan to humans has been well shown in a patient at the Mayo Clinic with islet cell caroinoma and extensive metastases (Wilder, 1946). Alloxan was used as a last resort but after three injections the patient developed acute yellow atrophy and died with extensive liver necrosis.

In view of published reports the use of alloxan in cases of hyperinsulinism cannot be advised because of the dangerous toxic effects that may accompany its use, the uncertain clinical results, the suggestion that islet cell tumors may be more resistant to alloxan than normal islet tissue, and because surgical removal of a pancreatic adenoma in competent hands offers a cure with much less risk.

IX. SUMMARY

The chemical alloxan produces diabetes in many animals by a selective destruction of the beta cells in the pancreatic islets of Langerhans. With a suitable dose pathological changes in other organs are minimal and reversible. Alloxan is quickly destroyed or removed from the blood stream after its injection and its presence in the blood has not been definitely established except following its intravenous injection. The exact mode of action of alloxan is unknown.

Several substances, especially sulfhydryl compounds, will prevent diabetes if injected just before the alloxan is given.

Whether alloxan is related to human diabetes is not known but human islets of Langerhans have been destroyed and diabetes produced in at least one case by the injection of alloxan.

Alloxan affords a simple, feasible method for producing diabetes in the laboratory animal.

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The Chemistry of the Hormones of the Posterior Lobe of the Pituitary Gland*

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I. INTRODUCTION

Ten years ago the author published a review of this subject which covered the literature up to about 1937 (Stehle, 1938). Since the arrangement of material was chronological it is now possible to bring the literature up to date without involving any repetition of material or break in continuity. The reader may pass directly from the section devoted to the pressor and oxytocic hormones to this extension; the same holds with regard to the section devoted to the chromatophore hormones.

II. THE PRESSOR AND OXYTOCIC HORMONES†

The original review terminated with the work of Stehle and Fraser (1935) on the separation and nature of these substances. Shortly thereafter their data were extended by Stehle and Trister (1938). Proline and isoleucine were found to be present in the pressor substance and proline and leucine in the oxytocic. Tryptophan was present in traces only in both hormones and histidine, glutamic acid, phenylalaline, hydroxyproline and glycine could not be detected under conditions which should have led to their detection had they been present.

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* In this and the following review the term "posterior lobe" is used to mean that portion of the pituitary gland which is separated from the anterior lobe by the cleft. It, therefore, includes the pars intermedia and the pars nervosa.

† The only oversight the author has noticed in the original review concerns several papers published by Gulland and his collaborators. Gulland and Macrae (1933) have described the behavior of the oxytocic hormone toward several enzymes. They were led to the conclusion that enzymatic destruction is not due to hydrolytic cleavage but to the action of other enzymes present in the enzyme preparations used. Thus the evidence did not favor the idea that the hormones were polypeptides. Freeman *et al.* (1935) were unsuccessful in attempts to purify the oxytocic hormone by various adsorption technics. Alone (1933), and with Randall (1935a, b), Gulland has also studied the actions of many chemical agents upon the oxytocic hormones.

Irving *et al.* (1941) have applied electrophoresis in the purification of the pressor hormone, an earlier investigation by du Vigneaud *et al.* (1938) having shown that both hormones migrate toward the cathode, the pressor substance about six times as fast as the oxytocic. They first applied the method of Kamm *et al.* (1928) (as described, but also using "unpublished data concerning the fractionation of the posterior lobe hormones supplied by Kamm") to obtain as potent a preparation as possible by chemical means. Their only thought concerning the oxytocic hormone seems to have been to get rid of it. The product obtained, which amounted to 120 mg. from 1 kg. of posterior lobes (frozen?) assayed 125 pressor and 20 oxytocic units per mg. From this, by electrophoresis, 40 mg. of material with 198 pressor and 26 oxytocic units per mg. were obtained.

In a preliminary communication published in 1941 Potts and Gallagher announced a high degree of separation of the oxytocic from the pressor hormone by an adsorption process. In 1944 they described the methods in detail. They found that the pressor hormone was adsorbed from a simple aqueous extract to a greater extent upon permutit than was the oxytocic, and by repeating the process they were able to obtain solutions in which the ratio of pressor activity to oxytocic activity was 20:1. They described no preparation in which the pressor hormone was freed to the same extent from the oxytocic. Between the preliminary communication and the detailed paper the same authors wrote a Letter to the Editor of the *Journal of Biological Chemistry* (1942) containing some very interesting data, analyses of preparations more potent than anyone has hitherto described (oxytocic potency 350 times standard powder, pressor potency 225 times standard powder). Perhaps the most interesting feature of the results is the inference that the oxytocic hormone probably contains no arginine. Unfortunately the letter contains no details of preparation. The paper of 1944 contains references to this letter but in no way amplifies it.

The inference to be drawn from the results of Potts and Gallagher is supported by some recent results of Fraser (1949). He found, using the oxytocic and pressor preparations of Stehle and Fraser that arginase destroyed about half the arginine present in the oxytocic hormone without affecting its oxytocic activity and had no effect upon either the arginine content or activity of the pressor hormone. Tyrosinase destroyed both the tyrosine and pressor activity of the pressor hormone and about 75% of the tyrosine and 90% of the activity of the oxytocic hormone. The results with tyrosinase seem to indicate that part of the tyrosine in the oxytocic hormone is not susceptible to tyrosinase action. The fact that the activity is more affected than the tyrosine content may indicate that tyrosine present in impurities is not susceptible to tyrosinase action.

Table I gives a summary of the analyses of the pressor and oxytocic preparations of various investigators. Inspection of the data will show that these cannot be reconciled without making assumptions concerning impurities which must be present in the less active preparations, assumptions which cannot be tested satisfactorily.

The possibility that, aside from the melanophore action, the other actions of posterior lobe extracts may be due to a single molecule has recently been revived by Van Dyke *et al.* (1942). If the separate oxytocic and pressor hormones described by others are split products of a single molecule it should be possible, by employing sufficiently mild concentration methods, to isolate the unsplit molecule. With this end in view heating was avoided, the pH did not exceed 3.5 in any of the purification stages, and the only reagents used were sodium chloride as a precipitant and phosphate as a buffer. Precipitation was repeated until the amount of nitrogen remaining in solution reached a constant value. Information obtained with the aid of electrophoresis and of the ultracentrifuge also indicated chemical purity. When dissolved and reprecipitated with sodium chloride the material remaining in solution and the precipitate had the same potencies. The molecular weight was found to be about 30,000. The product was eight times as active as standard powder, from which it follows that one-eighth of the posterior lobe is hormone.

TABLE I

Substance	Potency[1] units per mg.	N	S	Cystine (Sullivan method)	Tyrosine	Arginine	Investigators
Pressor	200 (8–10)[2]	13.86	2.95	7.73	9.53	8.78	Stehle and Fraser (1935)
	200 (?)		3.10	Trace	10.5		du Vigneaud *et al.* (1933)
	200 (25)	14.3		11.12	9.9		Irving *et al.* (1941)
	500 (40)			19.0	11.9	12.3	Potts and Gallagher (1942)
Oxytocic	250 (4)[2]	13.76	3.60	8.93	10.73	6.02	Stehle and Fraser (1935)
	500 (?)		3.1	9.0	14.3		du Vigneaud *et al.* (1933)
	700 (20)		5.6	18.3	14.2	0.8	Potts and Gallagher (1942)

[1] The figures in parentheses indicate pressor or oxytocic units, as the case may be.

[2] Not corrected for ash (pressor 3.47 %, oxytocic 4.50 %).

Van Dyke and his associates have made the best case yet for the single-molecule idea. Possibly one may be pardoned if one thinks their data may ultimately be explained in some other fashion. However, if that is merely caviling one may ask several questions. How are the results to be harmonized with those of others referred to above, which indicate that the two hormones do not occur in the same proportions in all species? Also, if the pituitary gland elaborates only one hormone with multiple activities does it necessarily follow that that is the form to which the physiological actions are due? There is evidence to the contrary in the paper of Van Dyke and his collaborators. At one stage in their method salt was removed from the active precipitate by dialyzing. The authors say "during dialysis of this precipitate activity which is apparently non-protein is lost." The raw material, therefore, in spite of the mild treatment it received, seems to contain the separate, more potent substances. There would seem to be an equilibrium between the parent molecule and the separate hormones in the solution. The splitting of the parent molecule appears to be as much a function of the gland as its elaboration. One can say with some measure of assurance that the hormones leave the gland as separate substances but with no assurance whatever that a parent molecule leaves the gland. The possibility that no splitting occurs in the gland during life would seem to be beyond the possibility of testing.

III. THE CHROMATOPHORE HORMONES

The action of alkali upon the melanophore hormone was studied intensively by Chen and Geiling in 1943. They showed that the potentiation reaction proceeds best when the pH is 11.2 and is complete in 3 minutes when the temperature is 100°C. The ratio of doses of the untreated and alkali-treated hormone which caused maximal melanophore expansion was 4:1. When equal doses were given the period of action of the alkali-treated hormone was about six times as long as that of the untreated substance.

In 1937 Böttger (1937a) described a method by means of which one may obtain a preparation of the "pigment hormone" fifty times as active as posterior lobe powder. It is based essentially upon the solubility of the hormone in ethanol, its adsorbability upon gallic acid, and its insolubility in ether. The potency stated is found in Böttger's summary. In the text he states that the preparation was 150–200 times as strong as posterior lobe powder on *Rana esculenta*. In a paper immediately following (1937b) his data show that 0.25 γ did not cause a maximal effect. (Since *Rana pipiens* responds to the reviewer's preparation to be mentioned later when the dose is only 0.005 γ it would seem to follow that data obtained from the different species are not directly comparable.) Böttger states that his preparation when assayed by his Phoxinus method was 50 times as potent as standard powder and explains the high potency found by the melanophore method in frogs on the assumption that crude extracts of the posterior lobe contain a substance which antagonizes the melanophore action but not the erythrophore action; the extraordinary potency found when the assay is made using *Rana esculenta* is therefore not real but is due to the removal of the antagonistic contaminant.

A very simple method of isolating a melanophore preparation from posterior lobe powder has been described by Landgrebe and Waring (1942). An aqueous extract of the lobes is treated with charcoal; this adsorbs the hormone, which may be eluted with phenol. When this phenolic solution is poured into ether the hormone precipitates. It is virtually free from the pressor and oxytocic hormones. Treatment with alkali prolongs the response. The data do not enable one to tell what degree of purification was achieved. Landgrebe *et al.* (1944) improved the method by substituting dilute acetic acid for water in the extraction of the pituitary powder, and by eluting with glacial acetic acid before precipitating with ether. This preparation was about 40 times as active as standard powder. A remarkable feature of the preparation was that it was not potentiated by treatment with alkali. A preparation (16–18 mg.) 330 times as potent as standard pituitary powder was obtained from 5 g. of pituitary powder if, before the adsorption step, the crude extract was made alkaline and heated at 100°C. for 10 minutes. It is obvious that there cannot be 16–18 mg. of such a potent substance in 5 g. of posterior lobe powder, so that the product must have been alkali-potentiated hormone. Consequently it cannot be said that the hormone had been concentrated 330 times. The product was a brown powder, containing nitrogen, soluble in water and hot alcohol, and insoluble in acetone and ether.

Abramowitz *et al.* (1943) have described a method for obtaining a solution of the melanophore hormone from whole pituitary glands.

In 1944 Stehle further purified the preparation described by him in 1936 by precipitation of the hormone with picric acid and subsequent removal of the picric acid. It was 73 times as active as standard powder and contained tyrosine (4.85%), arginine (4.84%) and cystine (about 3.2%). A little later (Stehle, 1945) the product was shown to contain tryptophan (5.7%). The pressor and oxytocic hormones do not contain tryptophan.

IV. CONCLUSION

In conclusion it may be observed that the slowness with which knowledge of the chemistry of the posterior lobe hormones has advanced is in striking contrast with the rapidity with which the chemistry of some other hormones and of vitamins has been elucidated. Two articles in this volume show how quickly the purification and chemical characterization of the anterior lobe hormones have been accomplished. The difference may be due to the more dramatic nature of the biological activities exerted by the anterior lobe hormones, a circumstance which has therefore attracted a larger number of active workers. Expense is to some extent a factor too: commercial posterior lobe powder is not a cheap article. Whatever the reason for the slowness of the advance, the problem of isolating the hormones seems like a soluble one. The substances already obtained are so potent that they may be regarded as containing relatively little ballast. Some additional earnest effort might bring the matter to a successful conclusion. The problem represents one of the most important gaps in chemical endocrinology.

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The Actions of the Hormones of the Posterior Lobe of the Pituitary Gland upon the Circulation and the Secretion of Urine*

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I. INTRODUCTION

The actions of pituitary extract upon the heart and blood vessels and upon the secretion of urine have been studied more intensively than any of its other actions. Both are due to what is usually called the pressor hormone. Up until now no important action upon the circulation in mammals has been demonstrated in the case of the oxytocic or melanophore hormone. The oxytocic hormone according to Holtz (1932) opens a sluice mechanism in the hepatic veins of the dog and according to Woodbury *et al.* (1944) causes a transient fall in blood pressure in man which is of cardiac origin.

The quantities of pressor hormone which influence the blood pressure and the secretion of urine are very small. Stehle (1934) has shown that in the case of a preparation 100 times as potent as standard powder 0.0006 γ/kg. causes a definite antidiuresis in dogs and a frequently used dose in the pressor assay using chloretonized dogs is about 0.15 γ/kg. According to the data of Shannon (1942) 0.00011 units/kg./hr. injected intravenously into a dog with diabetes insipidus reduced the urine secretion markedly: this dose would be equivalent to about 0.00055 γ of a preparation 100 times as potent as standard powder. Thus the pressor hormone is among the most potent of all biologically active compounds.

II. EFFECT UPON THE BLOOD VESSELS AND HEART

The discovery of the vasoconstricting action of pituitary extract was one of the results of the work of Oliver and Schaefer (1895) upon the actions of various glandular extracts upon the blood pressure which led to the discovery of adrenaline. For twenty-five years the study of this and other actions of pituitary extract suffered from the fact that it was impossible to state the strength of the preparations used in terms of any satisfactory standard. This may account for the slowness with which the various aspects of the circulatory action have become clarified, for it is doubtless true that the preparations used in the older work varied enormously in their potencies.

1. Isolated Blood Vessels

The results of different investigators who have studied the effects of pituitary extract upon isolated vessels are quite variable. It is possible that some of the conflicting data are due to failure to neutralize the extracts used before adding them to the bath.

The behavior of rings of arteries from various sources toward pituitary extract has been studied by a number of investigators. Pal (1908) found rings from the carotid, coronary, femoral, and mesenteric arteries of cattle to be constricted by pituitrin. The peripheral portion of the renal artery was dilated. De Bonis and Susanna (1909) observed constriction in the case of the carotid, crural, coronary and pulmonary arteries and the crural vein and Dale (1909) states that rings from the pulmonary arteries of dogs and goats were constricted. Cow (1911) with rings from various herbivorous animals observed constriction in the case of the carotid and renal arteries but the behavior of the coronary was variable—sometimes constriction occurred, sometimes relaxation. He could detect no action upon the pulmonary and intercostal arteries. In the splanchnic area Cow found that rings from the hepatic, gastric, and splenic arteries, taken close to the organ supplied, were dilated more than rings taken near the origins of the vessels. He was of the opinion that the splanchnic vessels as a whole are dilated by pituitary extract. Campbell (1911) found the coronary, carotid, superior mesenteric artery, and superior vena cava to be constricted but whether constriction or relaxation occurred in the case of the subclavian, pulmonary, and splenic arteries depended upon the composition of the Ringer solution employed. Renal artery strips were always relaxed. Rothlin (1920) could not confirm the results of Pal and Cow concerning the dependence of the response upon the portion of the artery from which the rings were taken. He never found pituitary extract to cause arterial relaxation. Franklin (1926) recorded slight constriction of the mesenteric vein of the sheep. Holtz (1932) found the perfusion rate through the mesenteric arteries of cats and dogs to be diminished.

2. Isolated Organs and Tissues

The results obtained by the perfusion of isolated organs lead to the conclusion that pituitary extract causes some degree of vasoconstriction in all organs and tissues.

a. The Coronary Vessels. Dale (1909) was the first to observe constriction of the coronary vessels in the perfused heart (rabbitt) using the Langendorff method. McCord (1911) perfused the portion of the heart supplied by the descending limb of the left coronary artery of the dog and observed pituitary extract to lower the perfusion rate. Wiggers (1911) stated that the perfused "rabbit's heart isolated from all nerve centers" pituitary extract slowed the rate of perfusion; he gave no data. The same was found by Campbell (1911). No certain action upon the coronary flow could be detected by Rabe (1912) when he perfused the mammalian heart by the Langendorff method with infundin. Sumbal (1912) reported the coronary flow of the heart of the tortoise (*Testudo groeca*) to be increased by infundin but Gruber (1926b) repeated the work on the terrapin heart (he does not name the species) and found coronary constriction to occur. Gruber attributed Sumbal's results to the latter's failure to take the acidity of commercial extracts into account. Gruber (1926a) also studied the influence of a number of commercial extracts upon the coronary flow in cats, rabbits, and rats. Unless the extracts were first neutralized they dilated the coronary vessels. After neutralization they caused constriction just as did a neutral extract of pituitary powder. Extracts that contained chloretone sometimes failed to cause constriction even after neutralization. Gunn's (1926) experiments show that a 1:400 dilution of pituitary extract decreased the coronary flow 70% in the cat's heart perfused by his modification of the Langendorff method. Anrep and Stacey (1927–8) found the coronary inflow in the dog's heart to diminish 75% using Anrep's anemometer method with the heart-lung preparation. Bodo (1927–8) cannulated the coronary sinus in the heart-lung preparation and observed diminished coronary flow. Häusler's (1929–30) work, showing that the coronary flow in the heart-lung preparation is diminished in diastole is evidence that the diminution is due to coronary constriction and is not secondary to an action upon the heart beat. Ross *et al.* (1930) and Rössler (1930), in experiments carried out for another purpose, also observed decreased coronary flow in the heart-lung preparation.

When pitressin became available Gruber and Kountz (1930a) studied this product upon the isolated rabbit's heart and concluded that it was "a much better coronary vasoconstrictor substance than is the whole extract of the pituitary gland." (In the whole animal Antopol and Rössler (1934) say that the results with pitressin and whole posterior lobe extract are essentially the same.) Stehle and Melville (1932) with a modification of the Langendorff method of heart perfusion showed that, in the rabbit, pituitary extract may stop the flow completely.

Holtz (1932) states that 0.3 unit halves the coronary flow in the rabbit's heart but he records an increase in coronary flow in the perfused cat's heart and states that pituitrin does not constrict the coronary vessels in this species. He seems not to have known of the work of Gruber (1926a) and Gunn (1926). His results may have been due to the acidity of his preparation. The constricting action of pituitary extract on the coronary vessels of the cat is also to be seen in some experiments of Melville (1933b).

b. The Pulmonary Vessels. That perfusion through the lungs is slowed by pituitary extract was shown by Dale (1909) (species not stated), Campbell (1911) (cat), and by McCord (1911) (dog). Rössler (1930) could detect no evidence of vasoconstriction in the perfused lungs of the dog when 0.3 cc. of pitressin was injected into the pulmonary artery. He suggested that Dale's results may have been due to a higher dosage. Neither did Holtz (1932) observe any evidence of vasoconstriction in the perfused lung of the cat.

c. The Vessels of the Brain. Dixon and Halliburton (1910) and Roberts (1923) found that the vessels of the brain are constricted by pituitary extract. The former stated that in the isolated perfused brain the flow is slowed for 1 to 1.5 minutes and then increases. The preparation used and the dosage were not mentioned. Roberts found that 0.5 cc. of pituitrin increased the pressure in the Circle of Willis. The results of Dixon and Halliburton were confirmed by Gruber and Roberts (1926). It is clear from some experiments of Heymans (1925) that the cerebral vessels of the rabbit are constricted by pituitary extract.

d. The Vessels of the Spleen. After pituitary extract a diminished perfusion rate was noted by Dale (1909), by Campbell (1911) and by McCord (1911). De Boer and Carrol (1924–5) showed that this was due to vascular contraction by showing that pituitary extract does not constrict the musculature of the spleen.

e. The Vessels of the Liver. Campbell (1911) reported slight contraction of the vessels of the rabbit's liver. McCord (1911) found the perfusion rate through the liver of the guinea pig to be reduced. Morita (1915) could detect no influence upon the perfusion rate through the frog liver. Lampe and Mehes (1927) observed a diminution in the perfusion rate and a decrease in volume when the cat liver was perfused through the portal vein with a 1:50 dilution of a commercial pituitary extract (physormon). The rabbit liver was less susceptible, and McLaughlin (1928) could also detect very little effect in the rabbit liver. Clark (1928) could detect very little effect by perfusion through the portal vein of the liver of the cat. Holtz's (1932) records, also for the cat, show a slight decrease in flow and a decrease in liver volume.

f. The Vessels of the Kidney. Several workers have recorded diminished perfusion rates for the kidney after the administration of pituitary extract—Dale (1909), McCord (1911), Wiggers (1911), Pentimalli and

Quercia (1912), and Solntzew (1928). The protocols of Starling and Verney (1925), and of Verney (1926) show that this occurs also in the heart-lung-kidney preparation. Campbell (1911) found that pituitary extract increases the perfusion rate in the sheep kidney.

g. The Vessels of Striated Muscle. One of the experiments in Oliver and Schaefer's (1895) original paper concerned the effect of pituitary extract in the perfused frog; decreased flow was stated to occur. Salvioli and Carraro (1908) perfused the hind limbs of dogs, rabbits, and cats and observed that posterior pituitary extracts from various mammals caused vasoconstriction. When a limb, except for the sciatic nerve, was separated from the rest of the animal and perfused the injection of the extract into the trunk did not affect the perfusion rate in the limb. Campbell (1911) observed constriction in the hind limb of the cat. Rabe (1912) could detect no effect upon the vessels of the hind quarters of the rabbit when he perfused them with infundin diluted 1:100, and Nogaki (1924) reported that pituglandol even causes vascular dilatation in the hind limbs of the frog. Rössler (1930) observed strong constriction when the hind legs of the dog were perfused with blood containing pitressin.

h. The Vessels of the Ear. On perfusing the isolated ear of the rabbit through the central artery Rischbieter (1913) found that the injection of 0.5 ml. of 1:100,000 hypophysin decreased the flow about 50%. Similar results were obtained by Rothlin (1920) and by Ssentjurin (1928). Denervation has no effect upon the action (Levinson and Essex, 1943).

i. The Vessels of the Frog's Foot. Krogh and Rehberg (1922) found that the capillaries of the web of the frog's foot are contracted by very dilute solutions of pituitrin. Drinker (1927), using a somewhat modified method, could not observe this effect even though much higher concentrations were employed. Asher and Schneider (1926) found that the arterioles of the web are more sensitive than the capillaries.

3. The Vessels of Organs and Tissues in situ

In the whole animal differential vascular susceptibility is evident.

a. The Intestine. Schaefer and Vincent (1899–1900) observed a decrease in the intestinal volume in cats. Clark's (1928) experiments, in which the pressure in the portal vein was found to be decreased, also provide evdience that the intestinal arteries of cats are constricted; he eliminated the possibility that the fall in portal pressure might be due to increased flow through the liver. Later he (1930) reported that the intestinal volume decreased and that the intestinal pallor may endure for nearly an hour after 0.1 cc. of pitressin. Larson (1938) has recently recorded a decrease in volume in the intestine which, however, is much less important quantitatively than is the decrease in volume of the spleen. Holtz's (1932) results show that in the dog the intestinal volume first decreases slightly then increases greatly.

b. The Liver. The liver volume in dogs, cats and rabbits is decreased by pituitrin, according to Mautner and Pick (1923). This was explained by them as due to splanchnic vasoconstriction. According to Holtz (1932), in the dog but not in the cat, it is due to the opening of a sluice mechanism near the junction of the hepatic and caval vessels by the oxytocic component of pituitary extract. Schwiegk (1932), using Rein's "Stromuhr" technique, observed decreased flow through the portal vein and increased flow through the hepatic artery in the dog.

c. The Kidney. The increase in kidney volume which frequently occurs after an intravenous injection of pituitary extract is a passive phenomenon, the consequence of strong splanchnic constriction, according to Backman (1917). In the eviscerated animal the kidney volume decreased. The experiments of Ozaki (1927) are in harmony with this idea. When the systemic rise of blood pressure was prevented by the use of a compensator, vasoconstriction occurred in the kidney. This took place also when the kidney was denervated. Schretzenmayr (1933–4) maintains that the vasodilatation is the result of the action of metabolites formed when the activity of the kidney is increased by pituitary extract. When he obstructed the kidney vessels for 5 hours to abolish kidney function and then injected pituitary extract vasoconstriction resulted. Richards and Schmidt (1924–5), by direct microscopic examination, have observed that pituitary extract stops the flow of blood through the glomeruli of the frog's kidney. Many investigators have studied the kidney circulation in connection with the diuretic-anti-diuretic action of pituitary extract. The results of such studies will be found in the section devoted to urine secretion.

d. The Lungs. Constriction does not occur in the pulmonary vessels if the pressure in the pulmonary artery may be taken as a safe criterion. Sharpey-Schafer and MacDonald (1926) have shown that this even decreases slightly while the arterial pressure rises. When they employed an extract of ordinary posterior lobe powder a rise in pressure in the pulmonary artery occurred. This was presumably due to histamine.

In the heart-lung preparation Bodo (1927–28) found that the cardiac dilatation caused by the extract could not be explained by constriction of the pulmonary vessels: no change in the pressure in the pulmonary artery occurred. Ross *et al.* (1930) have shown that in the cat, with a mechanical heart substituted for the animal's, pituitary extract does not cause the slightest change in pressure in the pulmonary artery even when the carotid pressure is doubled. Controls with histamine showed that the expected increase in pressure in the pulmonary artery could be elicited.

Holtz (1932), on the other hand, found in his experiments with cats that the pulmonary pressure rose, but in the case of rabbits and dogs it decreased. To explain the rise in pressure in the pulmonary artery in cats Holtz suggested increased outflow from the coronary sinus. However, his results, both as concerns the effect of pituitary extract upon the pressure in the pulmonary artery and the effect upon coronary flow, are not in accord with the results of others.

e. The Brain. Yamakita (1922) observed the outflow from the temporal vein of the rabbit to decrease, and occasionally to cease, during the rise of blood pressure produced by pituitary extract. Miwa *et al.* (1928) found that blood flow through the brain and the pressure in the Circle of Willis increased after an injection of pituitary extract. If a blood pressure compensator was used, however, the blood flow decreased, though the pressure in the Circle of Willis increased as usual. By direct observation of the pial vessels Forbes *et al.* (1933) usually observed dilatation when pituitary extract was applied directly or injected intravenously. With the "thermostromuhr" Schneider and Schneider (1934) found the carotid flow to be diminished indicating constriction of the vessels of the brain. Simultaneously Geiling *et al.* (1934) with the same technique obtained similar results, but the doses of pressor hormone employed were large enough to cause sufficient cardiac depression in the unanesthetized dog to affect the circulation rate. The results of Schneider and Schneider are not subject to this criticism.

f. The Spleen. According to Schwiegk (1932) pituitary extract causes decreased flow through the splenic artery and vein.

g. The Muscles. Rosenow (1920) and Sacks (1924) record an increase in limb volume in man. This probably represents passive dilatation of the limb vessels: slight concomitant increases in blood pressure were observed by Sacks. Passive dilatation of the limb vessels is also indicated by Clark's (1930) experiments; if a blood pressure compensator was used an increase in limb volume did not occur, though it occurred when the blood pressure rose.

h. The Skin and Mucous Membranes. Attention has often been called to the pallor which follows the injection of pituitary extract—Behrenroth (1914), Beco (1914) Anderes and Wächter (1922–3) and Sacks (1924). Anderes and Wächter state that blanching does not occur during pregnancy. Sollmann and Pilcher (1917) scarified the skin in human subjects and applied pituitary extract to the abrasion. Blanching occurred which lasted about 30 minutes; no wheal formed nor did goose-flesh appear. Gröer (1918–19) suggested the use of local blanching, caused by the injection of the extract into the shaved skin of the guinea pig and rat, as a crude method of assay. Carrier (1922) found that injection at the base of the finger nail caused capillary but not arteriolar constriction. The skin vessels appear to be extremely sensitive, for Sacks (1924) states that 0.025 ml. of infundin intravenously in man causes noticeable pallor which lasts from 20 to 50 minutes. The vessels contracted were the venules and the capillaries. Killian (1925) believes that the arteriole-capillary valves are closed. He could observe this in the frog's tongue: the arterioles and capillaries were also constricted.

i. The Heart. In the whole animal the only determinations of the effect of pituitary extract upon coronary flow are those of Morawitz and Zahn (1914) and of Dietrich (1933). The former observed diminished outflow from the coronary sinus of the dog by means of their newly devised sinus cannula and the latter observed diminished flow through the anterior coronary artery by means of Rein's stromuhr. Green *et al.* (1942) have reported the coronary inflow to be diminished.

4. The Rate and Strength of the Heart Beat

a. Isolated Heart. In the isolated rabbit heart perfused by Langendorff's method Hedbom (1898) states that a water-glycerol extract of the whole pituitary gland of the rabbit decreased the frequency and increased the amplitude. Cleghorn (1899) obtained similar results as concerns rate on perfusing the apex of the dog's heart with a glycerol extract of the infundibulum; the amplitude, however, seems to have been decreased. Dale's (1909) results were similar to Hedbom's. Rabe (1912) states, but gives no data, that infundin increased the rate and amplitude of the perfused rabbit's heart. In the perfused tortoise heart Sumbal (1912) found the amplitude of both the auricular and ventricular contractions to be decreased. Gruber (1926a) repeated Sumbal's work but using the terrapin heart; he found that if the pituitary extract was neutralized before use it caused no change in the amplitude of the beat. In the case of hearts from cats, rabbits, and rats Gruber (1926b) found the amplitude of the beats to be increased by a saline extract of posterior-lobe powder. Gunn's (1926) tracings of the isolated cat's heart show that pituitary extract (source not stated) had no effect upon the rate or amplitude. In the heart-lung preparation Bodo (1928) records definite dilatation. Holtz (1932) has found the amplitude of the perfused cat's heart to be increased by pitressin. Melville's (1933b) data show slowing to occur in both the rabbit and cat heart; the bradycardia appeared to be greater, the greater the coronary constriction.

b. The Heart in situ. In the first paper on pituitary extract Oliver and Schaefer (1895) wrote: "The inhibitory action upon the heart, which is characteristic of suprarenal extract when injected into a dog with intact vagi, is not observed with pituitary extract." Howell (1898), on the other hand, was as much impressed with the slowing of the heart as he was with the pressor effect. Cyon (1898a) also was impressed with the cardiac slowing his extracts produced, and suggested (1898b) that two active substances were present, a pressor substance and an inhibitory substance. It is difficult to evaluate Cyon's results for his extracts exerted very little pressor action and yet he states that the slowing lasted for hours. When Schaefer and Vincent (1899–1900) returned to the problem in 1899 they found cardiac inhibition to be an inconstant effect and thought it might be due to a direct action upon the heart. In his "Textbook of Physiology" Schaefer (1898) gives a tracing showing increases in auricular and ventricular amplitude during the rise of blood pressure. Salvioli and Carraro (1908) were unable to abolish the slowing completely with atropine or by vagotomy. The participation of both central and peripheral factors was thereby indicated. Wiggers (1911) could obtain no constant effect with pituitrin upon the heart rate but considered that, on the whole, depression of the rate and amplitude was the characteristic action. His extract, pituitrin, must have been very weak for the doses he employed, 2–4 ml., were large enough to have caused a profound effect. Lewis *et al.* (1911) observed slowing and irregularity of the heart "in most cases." The first example of a diphasic blood pressure effect (a tendency to rise, a fall, then rise again) is found in a paper by Klotz (1911). He noted that the rabbit heart was weakened during the fall of pressure and that the pulsations were much increased in amplitude during the second rise in pressure. Pankow's (1912) results were much like those of Klotz but he furnished some additional facts. In rabbits 1 ml. of pituitrin slowed the heart and the beat became very weak or practically disappeared for a time. During the period of cardiac weakness the blood pressure fell markedly. Vagotomy and atropine did not affect the result. Beyond stating that he thought the action of pituitrin to be on the heart Pankow made no attempt to explain the action further. Tigerstedt and Airila (1913) observed a decrease in the pulse rate and a decrease in aortic output of rabbits. The output remained low after the blood pressure had returned to the control level. They were familiar with the work of Pal (1908) and suggested that the cardiac depression was the consequence of coronary constriction. Hecht and Nadel (1913), in an electrocardiographic study of the effect of pituitrin in unanesthetized rabbits and dogs observed marked bradycardia. They were of the opinion that with thorough atropinization the slowing could be abolished.

Claude and Porak (1913) found that a defatted posterior lobe extract on intravenous injection into the rabbit or dog caused a fall of blood pressure and decreased amplitude of the heart beat followed by a slight augmentation of amplitude. A little later Claude *et al.* (1913a) stated that when injected intramuscularly in man, presumably the same material caused a rapid pulse lasting for about a minute and then marked slowing for not more than 1.5 minutes. The same authors (1913b) then studied the preparation electrocardiographically in rabbits and found the rate to decrease from 250 to as few as 60 beats per minute. Repetition of the injection was without effect. Vagotomy also made no difference in the result. Since at the beginning of the bradycardia they noticed a 2:1 rhythm they suggested that the extract had an elective action upon the His bundle.

Werschinin (1914) observed slowing during the rise of blood pressure caused by pituitrin in dogs and Niculescu (1914) states that he confirmed Pankow's observations on the blood pressure and cardiac changes induced by pituitrin and similar preparations; he also agreed with Cyon's observation that the bradycardia after a large dose may last for several days. Börner (1916) was the first to record a decrease in systolic force by means of the cardiac oncometer; dilation of the heart is perceptible in two of her tracings. By means of a technique too involved to describe here Müller (1917) found the right ventricle of the rabbit heart to be weakened by pituitary extract. Using a kind of mechanical plethysmograph Wolfer (1922) found both ventricles to be weakened and the heart slowed in the rabbit. Resnik and Geiling (1924–5) and Geiling and Resnik (1924–5) observed essentially the same effects with the electrocardiograph as had Hecht and Nadel but in addition a very brief acceleration of the heart occurred prior to the bradycardia. It made no difference whether Armour's pituitary extract or a pituitary "tartrate" of Abel and Rouillier was employed.* The bradycardia could not be abolished wholly with atropine. When the heart was watched by means of the fluoroscope Kolls and Geiling (1925) saw that it dilated definitely after the injection of pituitary extract. They also found that the pulse pressure was reduced and the minute volume halved.

Iwai and Schwarz (1924) found the minute volume of the dog's heart to be greatly decreased by pituitary extract; the circulation rate was calculated from the oxygen consumption and the oxyhemoglobin percentages of venous and arterial blood. They thought decreased venous return caused by peripheral vasodilatation would explain their results. Elimination of the splanchnic circulation did not affect the outcome but they went no further in seeking the region of the supposed vasodilatation. Clark (1929) recorded the heart beat of the vagotomized rabbit mechanically; slowing and weakness occurred during the dip of the diphasic blood pressure response. Gruber and Kountz (1930a) recorded the blood pressure and pulse of unanesthetized dogs with a membrane manometer and found that the cardiac slowing caused by pitressin could not be eliminated entirely with atropine. When no atropine was used the bradycardia was preceded by a period of tachycardia which coincided with the fall of blood pressure; bradycardia occurred during the subsequent recovery and rise. Vagotomy did not abolish the bradycardia but the heart beat was faster than in the nonvagotomized animal. If atropine was injected during the period of bradycardia, the vagi being intact, the heart accelerated. Ligation of the carotid arteries, to eliminate carotid sinus reflexes, plus vagotomy, did not prevent the cardiac slowing. The same authors (1930c) made an electrocardiographic study of the action of pitressin and obtained results similar to those mentioned above by those who have used this technic: the acceleration which preceded the slowing appeared to be more in evidence, however. Holtz (1932) states that 0.5 unit of pituitrin dilates the rabbit heart (oncometer). Goldenberg and Zdansky (1932) in a roentgenographic study which amounted to a repetition of the work of Kolls and Geiling on cardiac volume changes after pituitary extract confirmed the latter's results. Atropine did not change the outcome.

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* An account of the "tartrate" preparations is included in the article of Li in this volume.

5. *Interrelationship of Coronary Constriction, Cardiac Weakness and Rate*

From the foregoing data it is clear that pituitary extract diminishes coronary flow, weakens the heart, and slows it. Are the two latter effects the consequence of the first?

a. Coronary Constriction and Cardiac Weakness. It was Tigerstedt and Airila (1913) who first suggested that there might be a concentration between the coronary constricting action of pituitary extract and the cardiac impairment observed by themselves and by Pankow (1912). Kolls and Geiling (1925) suggested coronary constriction as a possible explanation for the cardiac dilatation which they had observed but thought that a direct myocardial action might also be a factor. Ross *et al.* (1930) sought to decide the issue by measuring the coronary flow and aortic output simultaneously in the heart-lung preparation on the assumption that if the former decreased before the latter it would be reasonable to conclude that coronary constriction was the cause of the cardiac weakness. They found the two phenomena to occur in the order stated. At practically the same time Rössler (1930) studied the same problem by taking the pressures in the auricles as criteria of cardiac impairment. He found the decrease in coronary flow to precede the rise of pressure in these two chambers. The connection between coronary constriction and cardiac impairment has also been supported by electrocardiographic data. Goldenberg and Rothberger (1931) found that the electrocardiogram after pitressin showed changes similar to those characteristic of cardiac asphyxia, changes in the T-wave being especially significant. Dietrich (1933) measured the coronary flow by means of the Rein stromuhr and at the same time recorded the beat electrocardiographically. High T-waves occurred when the ratio $\frac{\text{coronary flow}}{\text{work of the heart}}$ was reduced to one-third of the prepituitary value. The inhalation of 6% oxygen in nitrogen caused changes in the electrocardiogram similar to those caused by pituitary extract. Melville (1938) has recorded the blood pressure and taken the electrocardiogram simultaneously in unanesthetized dogs and reached the same conclusions as Goldenberg and Rothberger, and Dietrich. He found also that if coronary constriction is prevented by administering ephedrine before the pituitary extract the high T-waves characteristic of this action of pituitary extract do not appear.

b. Coronary Constriction and Cardiac Rate. Atropine or vagotomy seems at times to eliminate the cardiac slowing due to pituitary extract and at other times only to reduce the degree of slowing. It would seem reasonable to conclude, therefore, that the bradycardia is the resultant of two independent causes. The fact that vagotomy diminishes pituitary bradycardia implies that one of these courses is a reflex reaching the heart through the vagus. No peripheral vagus mechanism is involved. This type of slowing should occur principally when the dose of the extract is small enough to cause no cardiac impairment and is probably due to a reflex from the carotid sinus and aortic nerves. The bradycardia which remains after atropine and vagotomy may be assumed to be the consequence of coronary constriction sufficient to impair cardiac nutrition to the point where the rate as well as the force is decreased. This also can be eliminated, as two of Melville's (1938) experiments show, by vagotomy plus the prior administration of ephedrine. In one of these experiments the dose of pituitary extract was 10 units per kg., yet no decrease in rate was noticeable.

6. The Blood Pressure Curve

The vasoconstriction produced by pituitary extract appears to be due to a direct action upon the vascular muscle. No nervous elements seem to be involved. That the central nervous system plays no role is shown by the occurrence of vasoconstriction in perfused organs; that gangliomic stimulation is not involved is shown by its occurrence after nicotine (Airila, 1914), and that a peripheral nervous mechanism is not involved is shown by its occurrence after ergotoxine (Dale, 1909).

When a highly purified pressor hormone preparation is injected intravenously into an anesthetized mammal the response will be either of two types, the result depending upon the dosage. Figure 1 is illustrative. The simple pressor effect is obtained when the dosage is not sufficient to cause enough coronary constriction to interfere with the work of the heart. The diphasic response results when coronary constriction is sufficient to impair cardiac function. Good examples of this type of response are scattered through the literature. Papers by Klotz (1911), Pankow (1912), Führer (1913) and Roberts (1923) show it in the rabbit, others by Jackson and Mills (1919–20), Sharpey-Schafer and Macdonald (1926) and Raginsky *et al.* (1930) show it in the dog, and still others by Schaefer and Herring (1908), Stehle (1929) and by Larson (1936) show it in the cat.

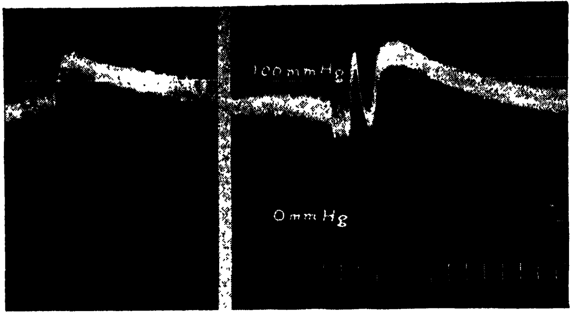

FIG. 1. Dog. Chloretone anesthesia. (A) Intravenous injection of 0.005 mg. of pressor hormone (postlobin-V). (B) 0.05 mg. same 30 minutes later.

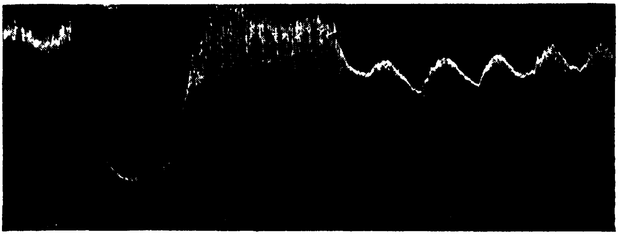

FIG. 2. Dog. Cannulation of femoral artery under local anesthesia. 0.3 ml. vasopressin intravenously. (Gruber, C. M. 1929. *J. Pharmacol. Exptl. Therap.* **36,** 155.)

When the injection is made into an unanesthetized animal the coronary constriction is so severe that the depression of the blood pressure dominates the record. Figure 2 from a paper by Gruber (1929) is the first published example of this action. Other instances are recorded by

Goldenberg and Rothberger (1931) and Melville (1933). It may be that the coronary effect is less marked in the anesthetized animal because of an antagonism between pituitary extract and anesthetics upon blood vessels—chloretone, for example, causes coronary dilatation.

In the literature of pituitary extract other types of blood pressure response may be seen. Figure 3 from a paper by Sharpey-Schafer and Macdonald (1926) is an example. In this case the variations were caused by the contamination of the preparations with a depressor substance or substances. If the contaminant is contained in sufficient quantity its presence is shown by the first injection. A fall of blood pressure occurs first and is followed by the pituitary hormone effect. If the amount of depressor substance is small its presence may be entirely obscured in the first effect but is manifest after subsequent injections. Abel and his collaborators have always maintained that the depressor effect observed on repetition of an injection is due to the pressor hormone; they reject the explanation given, but the explanation offered by Geiling and Campbell (1926) that "the inversion effect obtained by repeated injections of extracts of the pituitary body is due, in part at least, to the alterations which have occurred in the whole cardio-vascular system of the animal as a consequence of the original administration" is too vague to be satisfactory. The conditions which Geiling and Campbell found to be

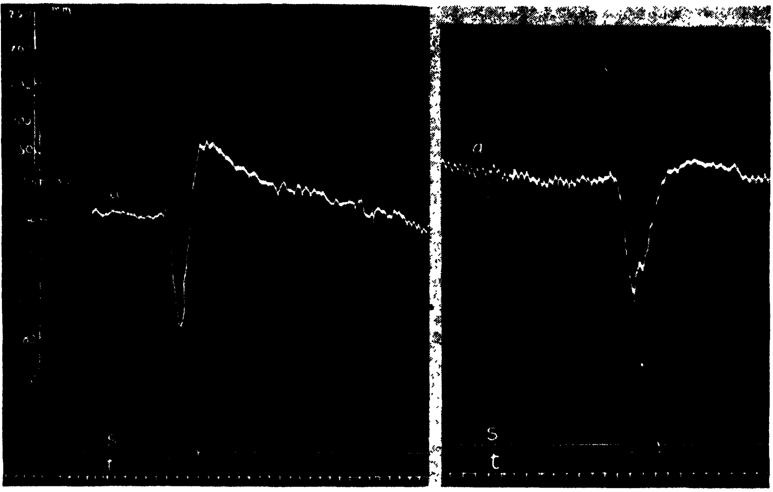

Fig. 3. Cat. Chloralose anesthesia. (A) Five milligrams of posterior lobe of ox pituitary, not previously extracted with alcohol. (B) Repetition of same a few minutes later. (Sharpey-Schafer, E. A., and Macdonald, A.D. 1927. II. *Quart. J. Exptl. Physiol.* **16**, 251.)

best for demonstrating the inversion effect, a small initial dose followed by a large dose given a few minutes later, are ideal for demonstrating contamination with a depressor substance, for, by making the large injection during the period of refractoriness, the presence of the depressor substance is no longer obscured. Many investigators, following Schaefer and Vincent's discovery that the reversal of action could be abolished by thorough extraction of posterior lobe powder with alcohol, have obtained preparations which exhibited no reversal on repeated injection. The papers of Hogben and Schlapp (1924) and of Larson (1936) directed at this particular point may be mentioned specifically. In addition Dudley's (1923) rather highly purified pressor fraction caused no reversal nor did the highly purified pressor preparation of Kamm *et al.* (1928). It was also shown by Stehle (1929) that a pituitary powder (obtained by extraction of posterior lobes with water, concentration of the extract and

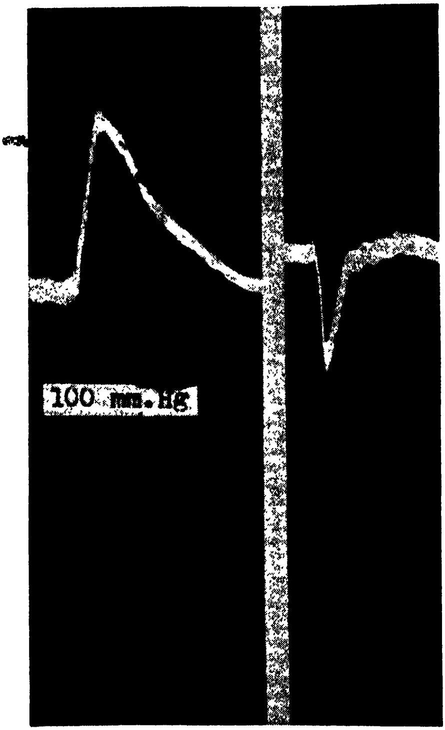

FIG. 4. Cat. Ether anesthesia. (A) Four milligrams alcoholic precipitate from a posterior lobe extract + 0.0033 mg. histamine bihydrochloride. (B) Same 10 minutes later. (Stehle, R. L. 1929. *Am. J. Physiol.* **88**, 724.)

precipitation with alcohol) had no depressor action on repeated injection, but when a minute quantity of histamine was added to the solution of this powder a reversal effect could be demonstrated clearly (Fig. 4).

7. *The Circulatory Action in Birds*

Paton and Watson (1912) were the first to determine the effect of pituitary extract upon the blood pressure in birds. A decrease followed the injection of 0.5 ml. pituitrin in the duck. The amplitude of the heart beat increased but the rate showed little change. Dilatation of the abdominal vessels accompanied the fall of blood pressure. Paton and Watson were of the opinion that the cardiac effect was due to a direct action of the extract. The depressor effect in the duck was confirmed by Hogben and Schlapp (1924) and shown to be true for the fowl too. However, both groups of workers sometimes obtained mixed effects—a decrease in pressure followed by an increase. A tracing published by Gibbs (1928) shows this mixed effect too. As soon as the separated pressor and oxytocic hormones became available Gaddum (1928) compared them on the blood pressure of the fowl and concluded that the depressor effect was due to the oxytocic substance. He observed no pressor effect with either preparation. Morash and Gibbs (1929) performed similar experiments soon after Gaddum and confirmed them with respect to the oxytocic hormone, but found that the pressor hormone caused a fall of pressure followed by a rise. Holtz (1932) found the avine depressor action of pitocin to be about 20 times that of pitressin, which is what it should be since 20 units of pitressin are contaminated by about 1 unit of pitocin. Werle and Hoff (1938–9) attempted to fractionate the posterior lobes of the pituitaries of chickens by the method of Kamm *et al.* Possibly because the quantity of raw material was small they did not obtain precipitates when they were expected and consequently had to use evaporation residues in their experiments. Both fractions lowered the blood pressure in chickens; the authors did not test their products in mammals.

8. *Effect of other Drugs upon the Response to the Pressor Hormone and Vice Versa*

According to Clark (1929), if rabbits are fed dried thyroid for a week or so they are then more susceptible to pitressin than are normal rabbits. Whereas no symptoms followed the intravenous injection of 0.2 ml. of pitressin before the thyroid period, after the thyroid period (which varied for different animals) they were killed by 0.2 ml.

The heart is sensitized to pituitary extract by luminal. Raginsky and Stehle (1932) have shown that in the heart-lung preparation, when the blood contains 25 mg. % of luminal, the lethal dose is less than one-tenth the dose which alone causes only temporary embarrassment.

The coronary constricting action of pituitary extract may be antagonized by a number of substances which dilate the coronary vessels. In the heart-lung preparation Melville and Stehle (1931) have shown that if ephedrine or adrenaline is injected into the inflow tubing before a dose of pituitary extract which alone causes a great decrease in cardiac output no decrease in output occurs. In the whole animal their experiments also show that the diphasic blood pressure curve which follows a large dose of pituitary extract is modified so that something more like a pure pressor effect is noticed.

In the unanesthetized dog, Melville (1933a) has demonstrated that the same two substances as well as nitrites prevent the severe depressor action. On the coronary vessels of the rabbit's heart he found (1933b) that the antagonism between the pituitary extract on the one hand and ephedrine, adrenaline or nitrites on the other hand is evident but it is not complete. Whole pituitary extract according to Gruber (1929) does not exert the severe depressor action in unanesthetized dogs which the pressor hormone alone exerts. Melville (1936) has shown that this is true also for a mixture of the pressor and oxytocic hormones, but in the heart-lung preparation he could obtain no evidence that the oxytocic hormone antagonizes the coronary constricting action of the pressor hormone. The phenomenon is without explanation at present.

Mautner and Pick (1929) state that the previous administration of morphine hinders the coronary constricting action of pituitary extract. However, the blood pressure curves in their figures are so atypical of pituitary extract effects that their conclusion requires verification.

The work of Antopol and Rössler (1934) shows that after papaverine a pure pressor effect is observed.

That pituitary extract sensitized various tissues to adrenaline was claimed by Kepinow (1912). When the blood vessels of the frog were perfused with a pituitary extract solution so dilute that it itself caused no diminution in the perfusion rate the minimal concentration of adrenaline which would then cause vasoconstriction was only a fifth as large as that required before the pituitary perfusion. The effect of an adrenaline injection on the blood pressure of the rabbit was also greater if it was preceded by an injection of pituitary extract.

Rischbieter (1913) could not detect any sensitization to adrenaline when isolated rabbit's ear was perfused with hypophysin. Kepinow's results on the blood pressures, however, were confirmed by Niculescu (1914) and by Airila (1914). Börner (1916) offered another explanation for this effect. She was of the opinion that it was a consequence of the diminished circulation rate caused by the extract. The concentration of adrenaline in the blood flowing through the vessel into which the injection was made would be higher than when the circulation rate was high and hence would cause a greater vasoconstriction.

9. *The Refractory State*

Unlike the pressor effect of adrenaline the pressor effect of pituitary extract cannot be duplicated soon after the previous blood pressure level has been attained. For a time a state exists in which the response to the initial dose is reduced. It is generally assumed that when the original blood pressure level has been restored the pituitary effect has worn off, and that because of some obscure state of affairs the blood vessels are incapable of responding in normal fashion for a time. This has by no means been proved. It is not beyond the range of possibility that the return of the blood pressure to the normal levels is merely the result of compensatory mechanisms and that the vascular state caused by the injection persists, as has been suggested by Larson (1938). If this is true then it is not surprising if a second injection is less effective than the first. Tigerstedt and Airila (1913) state that in their experiments the cardiac output never reached the pre-pituitary rate. The coronary flow in the experiments of Ross *et al.* (1930) was slower at the termination of their observations than at the beginning though the cardiac output was again practically normal; the periods of observation were short, however. Kolls and Geiling (1925) state that the mucous membranes of the dog may be still visibly blanched 1.25 hours after pituitary extract. The explanation proposed by Larson is not entirely satisfactory, for, in the case of the heart-lung preparation, 3 hours after the cardiac output has returned to normal after having been severely reduced by pituitary extract a second injection is without effect (Raginsky and Stehle 1932).

III. EFFECT UPON THE SECRETION OF URINE

1. *Introduction*

Any discussion of the effect of pituitary extract upon the secretion of urine suffers from the incomplete state of knowledge concerning the kidney mechanism and the mechanism by which water balance is maintained. This is not the place to discuss either of these problems. When authors interpret their results according to the filtration-reabsorption theory of urine secretion (which is often done) it must be kept in mind that the evidence for this theory in the case of the mammal rests upon indirect evidence, and that as more knowledge accumulates the concessions which have to be made to the theory are more numerous. At present it must even be admitted that the excretion of some substances cannot be explained upon the basis of the original theory; it is admitted that they are excreted by an active secretory process. It would appear rather daring, therefore, to be at all dogmatic about the mechanism involved in the action of pituitary extract upon kidney function. So far as water balance is concerned it must be admitted that there are no theories which have general acceptance.

Since this review is limited to the actions of posterior lobe extracts those studies are excluded which concern the role of the pituitary gland in regulating urine secretion and water balance. The reader will, therefore, find nothing about the effect of gland extirpation upon the secretion of urine and of variations in the antidiuretic action of blood and urine which are assumed to result from variations in the activity of the pituitary gland.

The action of pituitary extract upon the secretion of urine is peculiar in that an increase or a decrease in the rate of secretion, depending upon the circumstances, may follow its administration. If the rate of secretion is low, as is likely to be the case in anesthetized animals or in nonanesthetized animals deprived of water for a few hours, pituitary extract causes a slight and short-lasting increase in the rate. If the rate of secretion is high, when the extract is administered a diminution in the rate ensues. In either case the effect is preceded by a short period of anuria or marked oliguria if the dose employed is about 0.05 pressor unit or more per kilogram (intravenously in the dog).

The experimental work on these actions, to be discussed below in detail, leads to the conclusion that both actions are due to a single substance, the pressor hormone. However, a diuretic effect of the oxytocic hormone has been observed in recent years which is apparently independent of the diuretic action of the pressor hormone. It can be observed only when oxytocic preparations, freed as much as present methods permit from the pressor hormone, are employed; it seems to be wholly obscured in the presence of the pressor hormone. Aside from a piece of suggestive work by Draper in 1929 which has received no notice, until the work of Fraser in 1937, no clear-cut action of the oxytocic hormone on urine secretion had been demonstrated. The discussion of this matter will be found at the end of this section.

It is sometimes stated that pituitary extract causes diuresis in anesthetized and antidiuresis in unanesthetized animals. There is ample evidence, however, that antidiuresis may be produced in anesthetized and diuresis in unanesthetized animals.

Pituitary diuresis may be regarded as a special overdosage phenomenon which appears only when the rate of urine secretion is low, and which is the result of the action of the extract upon the excretion of salts.

Chronologically the discovery of the diuretic action of pituitary extract by Magnus and Schaefer (1901) antedated the discovery of the antidiuretic action by Velden (1913) and by Farini (1913). (The protocols in the respective papers show that Velden first used pituitary extract in diabetes insipidus on April 16, 1913, and Farini on April 26, 1913. Velden's work was read at a Congress on September 22 and Farini's was published on September 11.)

The original discovery of Magnus and Schaefer may be said to have delayed the second for it afforded a basis for a theory of diabetes insipidus which made a trial of pituitary extract in that disease pointless. Schaefer and Herring's (1908) experiments indicated clearly, as they pointed out, that diabetes insipidus might be due to pituitary hyperfunction, and the work carried such weight that Frank, as late as 1912, in a paper on the relation of the pituitary gland to diabetes insipidus, decided in the case he cites that the gland was hyperactive when the opposite conclusion now seems more logical. To Velden the facts were not so simple. The polyuria that followed puncture in the pituitary region and hypophysectomy was out of line with Schaefer and Herring's theory and he thought it desirable to observe the effects of pituitary extract in diabetes insipidus and in normal human subjects. Antidiuresis occurred. The daily urine output in his case of diabetes insipidus diminished from nearly 7 liters per day to less than 3 liters.

2. *The Diuretic-Antidiuretic Hormone*

Unassailable evidence that the diuretic-antidiuretic action of pituitary extract is due to the pressor hormone depends upon the isolation of the pressor hormone as a pure chemical compound. Preparations available at present may be merely mixtures of pressor and diuretic-antidiuretic hormones so similar chemically that their association is not affected by the procedures employed in the separation of the pressor and oxytocic hormones. The best that can be done toward a solution of the problem at present is to show that amounts of the purest pressor preparations thus far made and of simple posterior lobe extract, equal in pressor potency, have equal diuretic-antidiuretic actions. Evidence of this sort indicates that the diuretic and antidiuretic actions of pituitary extract are in all probability due to the pressor hormone. There is no occasion to think that separate diuretic and antidiuretic hormones exist.

In recent years some investigators have thought that they have obtained evidence that the melanophore hormone exerts an antidiuretic action, while others have thought that they have demonstrated the existence of an antidiuretic hormone separate from the pressor hormone, but neither of these views rests upon a secure foundation.

Draper (1927) furnished the first evidence that pressor and renal activity run parallel when he compared oxytocic and pressor fractions made from posterior powder by Dudley's butanol method.

Since the preparations of Kamm, Aldrich, Grote, Rowe, and Bugbee have become available others have investigated the problem. Bugbee and Kamm (1928) showed that the oxytocic substance exerted much less diuretic effect in the urethanized rabbit than did the pressor fraction. Draper (1929) obtained results which seemed to indicate that the oxytocic substance exerted more diuretic action in dogs than corresponded to its content of the pressor hormone. Hjort (1928) found the pressor fraction to have a much stronger antidiuretic action in man than did the oxytocic fraction. Burn (1928) used much smaller doses than Hjort and found the oxytocic fraction to have no antidiuretic effect. Later, however, Bijlsma *et al.* (1928), on the basis of suggestive but not conclusive assays of the pressor, oxytocic, and antidiuretic potencies of a number of commercial pituitary extracts, concluded that the antidiuretic action was due to a substance separate from the pressor hormone.

By comparing the actions of the pressor hormone with the oxytocic hormone upon water diuresis in man, Hemingway and Peterson (1929–30) found that the pitressin solution they employed was twenty times as potent as the oxytocic solution. They do not mention the strengths of the preparations employed but they refer to Burn's (1928) work from which it seems that the British ampules of the two hormones contained 25 pressor units and 12.5 oxytocic units per cc. Consequently Hemingway and Peterson's results indicate that 10 units oxytocic hormone were equivalent in antidiuretic potency to 1 pressor unit.

Zunz (1930) compared the antidiuretic potencies of ordinary pituitary extract, pitressin, tonephin, pitocin, and orasthin on water diuresis in dogs. The two oxytocic preparations were much less potent than the whole extract and the two pressor preparations.

Stehle (1934) could detect no difference between the antidiuretic activities of standard pituitary powder and postlobin-V and pitressin when the quantities administered were equal in pressor activity. Since the two latter preparations are made by entirely different methods, the result indicates that if the pressor and antidiuretic actions are due to separate hormones the chemical relationship must be so close that they have the same solubilities in a large variety of organic solvents. It was also shown that the antidiuretic activity of the oxytocic hormone was what was to be expected from the known content of the pressor hormone. Stehle's experiments also imply that the melanophore hormone has an antidiuretic action. The pressor preparation exerted practically no action upon melanophores and yet was equivalent to the standard powder, which contains the melanophore hormone, in antidiuretic activity.

The several authors who have attributed an antidiuretic action to one or another chromatophore hormone preparation give no information which enables one to determine how the preparation used was prepared. Zondek (1935), who states that intermedin definitely influences fluid output, but does not affect salt excretion, does not tell by which of his two methods of preparing intermedin the substance used was made. This makes a great difference. The preparation described by Zondek and Krohn (1932), which was made without the use of alkali, could be contaminated with the pressor hormone. The other preparation, made with the use of alkali, should have no pressor action. But which preparation was employed in the work which furnished the basis of his statement? It is only a statement, for no data are given. Sulzberger (1933) attributed an antidiuretic action in diabetes insipidus to an intermedin preparation furnished him by Zondek. Again there is no information about the particular preparation used. Later (1935) he concluded that it was not the intermedin but something else in the preparation which was responsible for the action. Dischreit (1935) states that intermedin (method of preparation not given) has no action in diabetes insipidus. Turner (1935) observed an antidiuretic action in diabetes insipidus to result from the use of intermedin prepared for him by Kamm, and from the use of a preparation called pitmelanin also made by Kamm. How these preparations were made it is not stated. Sulzberger (1936) could detect no action in diabetes insipidus from the melanophore preparation (pitmelanin) furnished him by Kamm.

To a pituitary preparation called "adiuretin" (method of preparation not given) Böttger (1936) has attributed an antidiuretic action. He regards "adiuretin" as a substance which on treatment with alkali gives rise to intermedin. Stehle (1937) has pointed out that Böttger's results can be accounted for by the presence of the pressor hormone contaminating his material.

The only determination of the antidiuretic potency of a chromatophore preparation by means of a quantitative method is that of Fraser (1937). Using a melanophore preparation described by Stehle, he found that the slight antidiuretic activity which it possessed could be accounted for by its content of the pressor hormone.

Though the above results seemed to indicate that the pressor and antidiuretic actions were due to the same hormone, experiments reported by Heller (1939) obscured the matter again. He found that if posterior lobe extract was brought to a pH of 0.57–10.0 and allowed to stand for a time the pressor activity diminished faster than the antidiuretic activity. His results were confirmed by Fraser (1941) but an explanation for Heller's conclusion that the two actions were due to different substances was provided. It was found that when the assay for antidiuretic activity was performed on dogs, the injections being made intravenously, no differential rates of destruction of the two actions could be detected. Evidently when posterior lobe extract is subjected to alkali, substances are formed which prolong the rate of absorption of the pressor hormone. Comparisons of untreated and alkali-treated extracts, in which the injections are made subcutaneously, afford no basis for estimating the ratio of the amounts of antidiuretic hormone in such preparations.

The most recent work bearing on the independence of the antidiuretic hormone is that of Donaldson (1947). He found that dialyzates had the same ratio of pressor to antidiuretic activity as the original solutions. At present, therefore, there is no convincing evidence which indicates that the antidiuretic action of pituitary extract is due to a hormone distinct from the pressor hormone.

3. *The Immediate Antidiuretic Action*

A frequent immediate effect of an intravenous injection of pituitary extract is anuria lasting up to 15 minutes. Schaefer and Herring (1908) and many subsequent workers have noticed this phenomenon. It may occur when the injection causes no fall in blood pressure. If the dosage is above 0.25 unit per kg. (dog) the fall of blood pressure resulting from cardiac impairment is in all likelihood a factor too.

Mackersie (1925) states that in urethanized rabbits this initial anuria is due to ureteral spasm; when a cannula was inserted into the pelvis of one kidney, diuresis from this kidney began immediately after the injection but no urine flowed from the bladder for several minutes. Unfortunately Mackersie gave no data, but McFarlane (1926) repeated these experiments and gives protocols which support Mackersie's statement. With a similar technique, but using the dog, Ross and Stehle (1930) could detect no difference in the rates at which urine was obtained from the pelvis of the kidney and from the bladder. One of Verney's (1926) experiments (Fig. 7 in Verney's paper) with the heart-lung-kidney preparation in which pituitary extract caused immediate anuria indicates that this was not due to ureteral spasm; according to the technique of the method very little of the ureter is retained and even that is probably functionless. Anuria does not follow the very small dosages (0.00025 units per kg.) which cause a marked antidiuretic action in the unanesthetized dog; the diuresis gives way to antidiuresis but no period of anuria is interposed. The immediate antidiuretic effect in the dog would seem to be due to a renal action, probably of a circulatory nature. The subject will be referred to later in connection with the kidney blood flow during antidiuresis. The antidiuretic action has been introduced in this place because it is part of the sequence of events met with in studies of the diuretic action.

4. The Diuretic Action

In a note published in 1901 Magnus and Schaefer provided the first evidence that extracts of posterior lobe of the pituitary gland, but not of the anterior lobe, exert an influence upon the secretion of urine in the anesthetized dog. The diuretic and the pressor actions did not occur concomitantly. The observation seems to have excited little interest, for no publications bore upon the subject until 1908 when Schaefer took up the problem again with Herring. They studied the effects of extracts of air-dried posterior lobes from cattle, made with boiling Ringer solution, upon anesthetized cats, dogs, and rabbits, and concluded that the diuresis was not due solely to the increases in blood pressure and in kidney volume because a second injection, though it increased the secretion of urine, was followed by decreases in blood pressure and kidney volume. They suggested that the posterior lobe contained a substance, different from the pressor and depressor substances present in the extracts, which exerted the diuretic action, and that the main purpose of the pituitary gland is concerned with the kidney—to augment urine secretion by direct stimulation of the renal epithelium. The idea naturally followed that diabetes insipidus might be due to pituitary hypertrophy. In a simultaneous but more restricted study of the problem Houghton and Merrill (1908) reached a different conclusion, namely, that pituitary diuresis is due to the rise of blood pressure caused by the extract.

Following these two papers other investigators took up the problem. The first efforts were made to determine whether the diuresis could be explained in terms of circulatory phenomena or whether an action upon the secretory processes of the kidney had to be assumed. The possibility of extrarenal factors being involved was not considered at once. When the antidiuretic action of the extract was discovered investigators in this field were dominated less by the vascular action and sought the explanation among a wider range of possibilities. Gradually it became apparent that there were features common to both the diuretic and antidiuretic actions. Hence in attempting to formulate the present states of either of these actions it is necessary to utilize data obtained in the study of the other.

The experiments of Halliburton *et al.* (1909), performed soon after those of Schaefer and Herring, with pituitary extract from human glands, showed that the diuretic effect in the cat runs parallel to dilatation of the kidney and increase in blood pressure. Hoskins and Means (1912–13) confirmed the work of Schaefer and Herring to the extent of observing that the diuresis may occur after the blood pressure has returned to the preinjection level. King and Stoland (1913) found the increase in kidney volume to run parallel with the diuresis. Consequently they thought vascular changes to account for the diuretic action. Knowlton and Silverman (1918) measured the blood flow during pituitary diuresis and thought that the increase observed accounted for the diuretic effect. Cushny and Lambie (1921) measured blood pressure, renal flow and diuresis simultaneously and concluded that "diuresis is the result of the larger blood supply to the kidney and not of any action on the secretory cells." McFarlane (1926) repeated and confirmed these experiments. Richards and Plant, (1922) determined the urine secretion, kidney volume, blood flow and blood pressure simultaneously and suggested that the increased kidney volume was due to constriction of the efferent glomerular vessels which, in conjunction with the slightly increased blood pressure, would increase glomerular filtration and consequently account for the increased urine secretion. Similar experiments were carried out by Livingstone (1927–8): he considered that his results confirmed those of Richards and Plant though in only three out of fifteen experiments was the proper coincidence of changes noted.

Those results which apparently contradict the secretory idea of Schaefer and Herring need not necessarily be assumed to do so. Even though increased blood pressure, increased kidney volume, increased blood flow through the kidney, and diuresis often occur concomitantly, it does not follow that the first three are responsible for the last. They may be incidental only. Certainly they cannot be the only factors, for then there is no explanation for the diuresis which occurs when they are lacking, the very thing which led Schaefer and Herring to suggest the secretory idea.

In the investigations cited the increase in the amount of urine secreted was all that was considered. It was assumed that urine was urine and that under the influence of pituitary extract the kidneys simply produced more of the same product. Had some attention been given to the composition of the urine it would have been realized that circulatory changes alone were not adequate to explain the phenomenon. The urine secreted after pituitary extract has certain characteristics which indicate that the action is more complicated than any circulatory theory implies.

On the other hand it does not follow either that if the diuresis is not due to circulatory effects it must be due to an action upon the renal epithelium. Extra-renal factors must also be considered.

Fromherz discovered in 1923 in a study of the antidiuretic action of pituitary extract that there was an increase in the absolute quantity of chlorides excreted. The chloride excretion had been studied previously in diabetes insipidus, but this effect had been missed because 24-hour urine outputs had been analyzed rather than the outputs for shorter periods following the injection. Two years later Stehle and Bourne (1925) showed that an increase in chloride excretion occurred also during the period of pituitary diuresis. In their studies of the action of pituitary extract in the heart-lung-kidney preparation Starling and Verney (1925) noted increases in the chloride excretion during the period of diminished urine secretion; these they believed to indicate a renal action of the extract. However the chloride increases noted were very small; in the first of their three experiments it amounted to 1.2 mg. per minute. In three similar experiments reported by Verney (1926), slightly decreased chloride excretions occurred in two and a slight increase in the third. Bayliss and Fee (1930), Hemingway and Peterson (1929–30), and Gremels (1929) have performed similar experiments. The maximum chloride increases noted were 1.8 mg. per minute.

Verney (1926) carried out a modification of the experiments of Starling and himself and drew the conclusion that the pituitary gland secretes a "chloride-raising-principle" with a direct action upon the kidney. The modification consisted in shunting the heart-lung-kidney circulation through the head of a second animal. This did undoubtedly diminish the volume of urine secreted and the decrease did not occur when the blood was shunted through the trunk of the second animal instead of through the head. Along with the antidiuretic effect an increase in the concentration of urinary chlorides occurred, but when the rates of chloride excretion in these experiments are calculated (Verney gives only the concentrations) it is found that in eight experiments three show decreased chloride excretion, one shows a slight increase and four are inconclusive. Thus there is no convincing evidence in Verney's experments that the pituitary has any effect upon the chloride-excreting mechanism of the kidney. The slight changes which have been reported are especially disappointing because in the intact animal the chloride excretion is increased by doses of pituitary extract much smaller than those which were employed by Starling and Verney under conditions which should have led to a very pronounced action if the action is renal. In the experiment cited 0.16 unit was added to the blood. The experiments of Unna and Walterskirchen (1936) show that the intravenous injection of .01 unit of pituitary extract raised the chloride concentration of the urine of a 9 kg. dog fivefold, and when the animal was on a chloride-rich diet 0.001 unit caused a definite chloride diuresis.

It should be recognized that the kidney in the heart-lung-kidney preparation cannot be in good physiological state; the urea excretion in the experiment of Starling and Verney amounted to only 1.6 mg. per minute even though the urea content of the perfusing blood was 80 mg. %.

In 1927 Stehle suggested that pituitary diuresis is what may be called a salt diuresis. According to this view the diuresis is the inevitable consequence of the increased salt elimination. There is a limit to the concentration at which salt can be excreted; hence if the task of eliminating an additional quantity of salt is imposed upon the kidney more water will have to be excreted too; in fact, the chloride concentration of the urine after the administration of pituitary extract rises while the rate of urine secretion is rising.

The diuretic effect of pituitary extract is increased, according to Melville (1936b), if sodium chloride is previously administered. This may also be seen in Silvette's (1939–40) experiments.

To say that pituitary diuresis is a salt diuresis does not explain it. The mechanism could involve the mobilization of tissue salts, the kidneys being passive, or it could be purely renal involving the secretion or reabsorption of chlorides. Because the Na:K ratio in the urine during the pituitary diuresis was quite different from the ratio in the prepituitary control Stehle inclined to the view that pituitary diuresis involved an extrarenal factor. The ratio in question changes from 5.2:1 to 0.51:1 in one of the experiments reported. McQuarrie *et al.* (1932) noted that pitressin caused a similar reversal of the Na:K ratio in an epileptic patient and McIntyre and Sievers (1933) have observed changes which were qualitatively the same but quantitatively of smaller magnitude in dogs. To explain these effects upon a renal basis requires that the extract have some selective action upon the potassium excretion, and there is nothing to support or eliminate this possibility. The alternative view, according to which the pituitary extract releases salts from extrarenal tissues, is a plausible one in view of the preponderance of potassium over sodium in the tissues. The release, however, may be indirect and unphysiological. It has been shown earlier that pituitary extract in high dosage dampens the circulation in the isolated kidney and it will be shown below that the ordinary antidiuretic action does not involve any diminution in the kidney blood flow. Since the vasoconstricting action is not confined to the kidneys it may easily be that the diuretic action is the reflection of a general or widespread tissue anoxia resulting in changes in cell membrane permeability and the passage of inorganic cellular constituents into the blood stream. Their elimination involves an increase in the rate of urine secretion. The composition of the blood serum should throw some light upon this hypothesis; McIntyre and Sievers could detect no definite change in either the potassium or sodium content of dog serum after injecting 20 units of extract intravenously but Yanagi's (1936) data show very definite increases in serum potassium.

What has been said concerning the excretion of potassium also applies to the excretion of phosphorus. Stehle and Bourne (1925) found it to be present in the urine in increased quantity during pituitary diuresis and Bollinger and Hartman (1925) and Gollwitzer-Meier (1926) observed that increases in blood phosphates occurred in dogs and rabbits respectively after the intravenous injection of pituitary extract. Fraser (1942) has found that this action is due to the pressor hormone and added the fact that the oxytocic hormone lowers the phosphate excretion. Tissues occur to one as a ready source of phosphorus, but on the other hand, those who prefer to regard the diuretic action of pituitary extract as of renal origin will explain its presence by some renal mechanism. Brull and Eichholtz (1926), in studying the effect of pituitary removal upon the secretion of inorganic phosphorus, concluded that the failure to secrete phosphorus is due to the absence of the regulating mechanism of the pituitary or of the wall of the third ventricle. However, they found that pituitrin produced "inconstant and doubtful effects" in the hypohysectomized dog or after injury to the tuber cinereum.

To bring the results of experiments with the heart-lung-kidney preparation into line with the extrarenal view of pituitary diuresis it is necessary to assume that the slight chloride increases which have been recorded are due to the relatively small tissue chloride reservoir in the preparation. This is, moreover, in harmony with the fact that those who have worked with the heart-lung-kidney preparation have never observed pituitary extract to cause diuresis. An intact, unanesthetized animal secreting urine at the rate the kidney was secreting in Starling and Verney's experiments would probably have responded to the extract by an increase in the rate of urine secretion. What actually occurred was a complete cessation of urine secretion in two of the three experiments, followed by a secretory rate about half that which was obtained before the extract. There is therefore some ground for thinking that since pituitary diuresis has not been observed with the heart-lung-kidney preparation it cannot occur in that preparation, and hence is of extrarenal origin chiefly—renal only to the extent that the three organs involved can liberate salts.

Nelson (1934) studied the diuretic action of pituitary extract in rabbits and reached the conclusion that the increased chloride excretion is due to increased glomerular filtration and diminished chloride reabsorption as the result of an accelerated flow through the tubules. The conclusion is open to question. He based his estimate of glomerular filtration upon the difference between the sucrose concentrations of the blood and urine during a continuous infusion of sucrose. In all likelihood what he assumed was diuresis due to pituitary extract was nothing more than the result of the pressor action of the extract (0.2 unit). One does not obtain diuresis by injecting pituitary extract into an animal secreting *normal* urine at the rate of more than 3 ml. per minute. One obtains an antidiuretic effect. The urine Nelson was obtaining before the injection contained more than 6% sucrose and was derived from highly diluted blood. A rise in blood pressure under such conditions might cause increased urine secretion. The increase in chlorides was probably directly due to the pituitary extract action but Nelson's data throw no light whatever upon their origin. The concentration (0.214%) is too low to account for the diuresis as a salt effect.

An interesting technique was employed by Miura (1925) to investigate the diuretic action of pituitary extract. In the rabbit it is possible to arrange things so that a substance reaches the left kidney first and directly while the right kidney receives it only by way of the general circulation. The results of Miura's experiments are, however, none too clear. He concluded that pituitary extract caused an initial antidiuresis by a direct action upon the kidney since the phenomenon appeared in the left kidney first. The antidiuresis he was observing, however, was the initial shortlasting anuria or oliguria and not the prolonged depression of a strong antiduretic action. As stated above, this immediate effect is probably due to some renal action or possibly to ureteral spasm. The diuresis which followed was stated by Miura to be due also to a renal action but this is plausible at best in only two of the four experiments for which he gives data. In one of the experiments diuresis appeared first in the right kidney and in another it appeared in both kidneys simultaneously. Janssen (1928) repeated Miura's experiment with a solution of hypophysin so dilute that the initial oliguria did not appear. In the protocol he gives, from the time of the injection until the diuresis was evident in both kidneys, there was a difference of only 3 drops of urine in favor of the left kidney (10 drops as against 7 drops). Since Miura's results show that the results are variable opinion must be reserved regarding the significance of the results with this technique until they have been repeated and more convincing data obtained.

5. The Antidiuretic Action

The antidiuretic action of pituitary extract is elicitable only when the diuretic urine is dilute as in the case of diabetes insipidus or after the administration of a large quantity of water.

Diuresis due to the ingestion of sodium chloride solution can not be abolished by pituitary extract as was shown by Frey and Kumpiess

(1914). Veil (1918) records data from a patient with diabetes insipidus who received physiological saline intravenously and continued to secrete urine at a high rate in spite of the repeated injection of pituitary extract. Brunn (1920) observed no antidiuretic effect in man upon diuresis caused by the ingestion of a liter of 2.5% sodium chloride solution. Molitor and Pick (1924) found that pituitary antidiuresis could be broken through by the administration of a strong solution of urea.

a. The Effect of Kidney Innervation upon Pituitary Antidiuresis. While only C. and M. Oehme (1918) appear to have investigated the effect of the nervous supply of the kidney upon pituitary diuresis (they observed a greater diuretic effect upon the denervated kidney), after the antidiuretic action was discovered the effect of denervation of the kidney upon the phenomenon was one of the first factors considered.

Motzfeld (1917) examined this effect but his results were inconclusive. The vagi are not involved in the antidiuretic effect for, after vagotomy, it was producible as usual. When the splanchnic nerves were divided the antidiuretic effect of the extract was usually observed but in two experiments it was not. Stripping the renal arteries likewise led to nothing decisive; usually this had no effect but sometimes the antidiuretic action seemed to be less marked than usual. McFarlane (1926) divided the right splanchnic nerve just above the adrenal gland and extirpated the left kidney. Pituitary extract still exerted its usual effect upon water diuresis. Fee (1929–30) administered pituitary extract to decerebrate hypophysectomized dogs in which one kidney was denervated by removing the outer coat of the renal artery, and found that the secretion of the denervated kidney was inhibited by pituitrin. Tuchman (1931) stripped the renal arteries, veins and ureters of rabbits and found that pituitary extract still exerted a strong anti-diuretic action. Samaan (1937), after the denervation of one kidney of a dog, could detect no difference between the degree of antidiuresis shown by the normal and denervated organs when doses of extract between 0.001 and 0.5 unit were administered.

b. The Effect of Pituitary Extract upon the Absorption of Water from the Alimentary Tract. The antidiuretic action of pituitary extract is not due to a delay in the absorption of water from the alimentary tract. Rees (1918) first reported that the extract practically stopped the absorption of water placed in intestinal loops. Later he (1920) arrived at the conclusion that the extract had a relatively slight action in this respect. Dixon (1923) states that the absorption of water from intestinal loops in dogs and cats was not affected by pituitary extract. Craig (1925) could recover practically no water from the alimentary tract of cats 2 hours after the subcutaneous injection of pituitary extract followed by 70 ml. water per kg. orally. Though the water was absorbed, very little secretion of urine occurred. Buschke (1928), thinking that pylorospasm might cause the retention of water in the stomach, tested the antidiuretic action of pituitary extract upon a dog provided with a gastroenteroanastomosis and found it unchanged. Heller and Smirk (1932) could detect no effect of pituitrin upon the rate of absorption of orally administered water in rats.

c. The Blood Flow through the Kidney during Pituitary Antidiuresis. While the data of those who have used the heart-lung-kidney preparation to study the antidiuretic action of pituitary extract contain many examples of a very definite diminution in blood flow through the kidney, none of the investigators has thought it of sufficient magnitude to regard it as a factor in the antidiuretic action.

The development of the thermostromuhr for measuring blood flow by Rein made possible the investigation of the antidiuretic action of pituitary extract under more nearly physiological conditions. Janssen and Rein (1928) found the extract to cause no change in the renal circulation of decerebrate dogs even though a strong antidiuretic action was produced. Handowsky and Samaan (1937), also using the stromuhr but giving the extract intravenously, found that large doses reduced the blood flow as well as the urine flow; small doses reduced the urine flow only. In the former case the blood flow returned to the normal rate while the antidiuretic action still persisted. Similar effects have been recorded by Walker *et al.* (1937), and by Wakin *et al.* (1942). These investigators observed, like Janssen and Rein, that when the injection is made subcutaneously the urine flow only is reduced.

The above review of studies of the circulation in the isolated kidney indicated that pituitary extract causes vasoconstriction. It is likely, therefore, that effects like those of Handowsky and Samaan and of others showing a reduction in the blood flow in the kidney *in situ* are due to a vascular action of high concentrations of the hormone. This action is not involved in the ordinary, perhaps physiological, antidiuretic action but is probably the cause of the "immediate" antidiuretic action discussed earlier.

An interesting conception of pituitary extract action has been suggested by Frey (1936). He found that when he injected Chinese ink during water diuresis it appeared in the subcortical portion of the organ, whereas if it was injected after the administration of pituitary extract it appeared in the cortical layer. The explanation suggested was that the extract causes the blood to be directed toward the glomeruli and away from the tubules. It will be recalled that according to Frey's (1932) minimal filtration theory of urine secretion diuresis is due to increased tubular secretion of water.

The facts of this and the preceding section seem to indicate that the antidiuretic action of pituitary extract in mammals occurs independently of the innervation of the kidney, of the rate of blood flow through it, and of gross changes in blood pressure.

d. Hydremia during Pituitary Antidiuresis. Data are not lacking which indicate that pituitary extract may have some action upon the kidney, apart from possible actions through nerve and blood supply, which may be the cause of the antidiuresis. These data concern changes in the composition of the blood and urine wrought by pituitary extract.

Konschegg and Schuster (1915) found the water content of rabbit blood to increase from 84.8% before to 86.2% after the administration of the extract. This dilution of the blood has been confirmed many times. Veil (1918) found the serum protein of men to diminish. C. and M. Oehme (1918) found that the hemoglobin and serum protein concentrations of rabbit blood decrease, but C. Oehme (1919) could detect no difference between the rates at which injected Ringer solution containing or not containing pituitary extract disappeared from the blood stream. Modrakowski and Halter (1919), in humans, found hemoglobin and serum protein to diminish and serum chloride to rise. The same changes occurred during water diuresis and when water was withheld. Underhill and Pack (1923) observed a diminution in hemoglobin percentage and Fromherz found the serum protein to decrease in dogs. In Underhill and Pack's experiments the blood dilution also occurred after the ligation of the kidney vessels. Frey (1925) infused Tyrode solution free from and containing pituitary extract and observed that in the latter case the blood volume at the end of the infusion was 253% of the control volume and in the former case 169%. After a half hour the difference had vanished; in both cases the volumes were still up about 20%. McIntyre and van Dyke (1931) found the water content of the serum to increase slightly in normal and in nephrectomized dogs. Molitor and Pick (1924) observed that the erythrocyte count decreased markedly in dogs but found that the count returns to normal before the antidiuretic action is finished. In man, Bauer and Aschner (1922) could detect no definite effect upon the serum protein and serum chloride, but Blumgart (1922) found the serum protein to decrease from 9.25 to 8.0% and Craig (1925) found the hemoglobin to decrease from 94 to 86%. Gollwitzer-Meier (1926) decided that the blood in rabbits become concentrated because she found that the oxygen capacity increased. Hines *et al.* (1927) injected salt solution intravenously and found that it remained in the circulation longer than normally. Poulsson (1930) found in man that the hemoglobin and plasma protein decrease more when pituitary extract is administered after the ingestion of water than when water is taken alone. Roboz (1931) reported that pituitrin extract caused hydremia in rabbits. Heller and Smirk (1932) also conclude that pituitrin causes greater blood dilution in the rabbit than water administration alone, but their data on this point are not wholly satisfactory; there is very little difference between the blood concentration in their control and pituitary experiments. The experiments of Gilman and Goodman (1937) are clearer on this point. They injected 200 units of pituitrin daily. The authors suggest that the anemia which accompanied the very marked hydration was due to the lowered osmotic pressure. Davis (1942) reports that a polycythemia develops gradually in dogs and rabbits when daily injections of 0.5–2.0 units of pituitary extract are made for 2–3 weeks.

With few exceptions the above citations show that pituitary extract causes hydremia, and they have been interpreted as supporting the theory that pituitary antidiuresis is through some renal action. C. and M. Oehme (1918) suggested that pituitary extract "diminishes the susceptibility of the kidney to hydremic stimulation." They were of the opinion that the secretion of water by the kidney was under nervous control and that in diabetes insipidus the activity of the water-secreting mechanism of the kidney is stimulated. In substance this amounts to saying that pituitary extract diminishes the secretion of water by the kidneys. At present the explanation of the nature of this renal action will depend upon whatever theory of kidney function one prefers. If Frey's "minimal filtration theory" is regarded favorably then one would regard the water secretion mechanism of the kidney as being depressed by pituitary extract. If, on the other hand, one accepts the filtration-reabsorption theory then increased tubular reabsorption of water must occur. A difficulty with the latter conception is that it requires increased reabsorption of water when the blood is already abnormally dilute.

The simple conception that pituitary antidiuresis is caused by kidney block (Nierensperre) meets with difficulties. Several of the investigations just cited show that hydremia occurs after nephrectomy and is consequently due in part at least to an extrarenal action, and Roboz (1931) and Yanagi (1936) found the hydremia was too great to be accounted for by the failure of the kidneys to secrete urine at the pre-pituitary rate.

The hydremia could be explained without resorting to the idea of "Nierensperre" if it could be shown that pituitary extract increases the osmotic pressure of the plasma proteins. This possibility has not stood the test. Oelkers (1931), Bonsmann (1932) and Yanagi (1936) have all found that the osmotic pressure per unit weight of proteins is not changed by the extract. Malkin (1930) and Tada and Nakazawa (1930) have reported that the osmotic pressure decreases.

e. The Antidiuretic Action in the Isolated Kidney. The experiments of Starling and Verney already referred to on the effects of pituitary extract in the heart-lung-kidney preparation are in a way the complement of the experiments on blood composition. The latter indicate that a renal factor is involved in pituitary antidiuresis and the former have been thought to provide direct evidence for this. The experiments of Starling and Verney led them to suggest that substances like pituitary extract normally regulate the output of water and chloride in the intact animal, and that the characters of the urine secreted by the isolated organ are due in large part to the absence of such substances. The fact that emerges clearly from these experiments and from those of Verney (1926, 1929) as well as of others who have performed similar experiments, is that in the heart-lung-kidney preparation pituitary extract by some mechanism (decreased secretion or increased reabsorption) diminishes the rate of water secretion. That the extract affects the secretion of chlorides by a direct action upon the kidney has not been at all clearly demonstrated.

Whether the antidiuresis exhibited by this preparation when pituitary extract is administered has physiological significance is doubtful. Bayliss and Fee (1930) regard the polyuria which develops in the heart-lung-kidney preparation as being due to the denervated state of the kidney and not to the absence of the pituitary hormone. When they modified the technique in such a way as to maintain nerve action they observed no polyuria and no cessation of chloride secretion. It seems impossible also to harmonize the results of Gremels (1929), which show a decrease in the rate of total nitrogen excretion from 3.36 mg. per minute to 0.29 mg. per minute, with results of others (mentioned in the next section) which show that glomerular filtration is unaffected. In Gremels' experiments the nitrogen excretion diminished almost as much as the water excretion. The rates of urea excretions in Starling and Verney's experiments were also decreased as much as was the water excretion.

f. The Effect of Pituitary Extract upon the Excretion of Nonthreshold Substances. Because the rate of excretion of so-called nonthreshold substances like creatinine, xylose, etc., is, according to some, not affected by pituitary extract, even when that of water is greatly diminished, the conclusion has been drawn that the antidiuretic hormone increases the tubular reabsorption of water. Obviously this conclusion is only valid if the filtration-reabsorption theory of kidney function is true. All that the experiments demonstrate is that pituitary extract diminishes the excretion of water more than it diminishes the excretion of some other substances.

Using the creatinine method for the calculation of the rate of glomerular filtration Poulsson (1930) found that during pituitrin antidiuresis glomerular filtration remains constant. According to him increased tubular reabsorption of water must occur. Iversen *et al.* (1934) with the same method but in a case of diabetes insipidus, found the rate of filtration to diminish for a short time after the administration of the extract. They thought their data showed that pituitary extract exerts a short vascular effect which diminishes the rate of filtration and that it also increases the reabsorption of water; they could see no evidence to indicate that it affected the reabsorption of chlorides since the chloride excretion ran parallel with the rate of filtration. Iversen and Bjering (1934) have noted the same decrease of filtration to occur in normal human subjects. Burgess *et al.* (1933) using the sucrose and xylose methods for calculating the rate of glomerular filtration have arrived at the same conclusion as Poulsson.

g. Pituitary Antidiuresis and the Central Control of Water Balance. The chief advocates of the extrarenal theory of pituitary antidiuresis have been Pick and his collaborators. In their first study Molitor and Pick (1924) arrived at the conclusion that the mechanism was not renal but they went no further in attempting to explain it. In their second study Molitor and Pick (1925a) observed that pituitary extract did not exert as strong an antidiuretic action when paraldehyde was given with the water as when water alone was given. The same was found when chloralose was used instead of paraldehyde. Failure of pituitary extract to cause antidiuresis was also observed in decerebrate rabbits. They thereupon wrote "es kann wohl keinem Zweifel unterliegen, dass die Ursache dieser Aufhebung in der Ausschaltung des Grosshirns durch die Narkose zu suchen ist." In a third paper Molitor and Pick (1925b) found that chloretone alone or superimposed upon paraldehyde narcosis caused antidiuresis. In this paper they proposed that a center in the region of the other vegetative centers supplies nervous or hormonal stimuli which regulate the water equilibrium of the organism. Paraldehyde was imagined to remove the damping influence of the cerebrum on the center which then became so hyperexcited that pituitary extract no longer exerted its characteristic action.

Molitor and Pick seem to have been undecided as to whether the water center controls the pituitary gland or whether the pituitary gland controls the water center. Their statement that the center exerts its action through nervous or hormonal stimuli implies the former view but in a later paper (1926) they state that "the secretion of the hypophysis is one of those substances which regulates the water center assumed to be located in the mid-brain." In the absence of the secretion the center was supposed to be hypersusceptible to the "Durstreize des Blutes" and to cause the tissue to give up water more readily. However, in a still later paper from Pick's laboratory, Hoff and Wermer (1928b) seemed to be of the opinion that the pituitary gland was under the control of the water center (nervösen Zentralstellen) via the supraoptico-hypophyseal tract. They found that the cisternal liquor of dogs excited by cats had a stronger oxytocic activity than the cisternal liquor of normal dogs. From this they inferred that the excitement had caused an outpouring of the antidiuretic hormone. They (1928a) also found that during diuresis produced by novasurol, euphylline, and urea the oxytocic activity of the cisternal liquor was increased. This they regarded as representing an effort of the water balance center to prevent the loss of water from the blood and tissues. No evidence was provided, however, that the blood had become concentrated.

Molitor and Pick (1925b) were no clearer on the subject of the antidiuretic action of pituitary extract. They thought it might have: (1) a direct action on the water center which in some unmentioned manner (presumably nervous) caused the tissues to bind more water, or, (2) that it might have a direct action upon the water-binding capacity of the tissues. The former possibility seems to have been preferred, for subsequently they (1926) described an experiment showing that the intralumbal injection of pituitary extract caused antidiuresis when the same dose administered subcutaneously had no effect. This view was adhered to three years later by Molitor and Nikoloff (1929) who reported a single experiment in which the intracisternal injection of pituitary extract caused antidiuresis as quickly as did an intravenous injection. (This was in confirmation of the work of Jánossy (1926) who had observed an extremely pronounced antidiuretic action in several human subjects when 1 cc. of undiluted pituitary extract was injected into the cerebello-medullary cistern. He could observe none when the extract was diluted 1:10,000.) They maintained that their result indicated the existence of a central mechanism or that the extract produced its effect through a peripheral but not renal action; they preferred the former view, apparently, because they were faced with difficulty of explaining how a substance could reach the blood from the cerebello-medullary cistern soon enough to cause as rapid an effect as that which follows intravenous injection.

That the water balance is controlled by nerve impulses originating in a water balance center was disproved by Janssen (1928). He could detect no change in the amount of urine excreted or in its specific gravity or chloride concentration when the cord was cut at the 5th cervical vertebra. That the antidiuretic action of pituitary extract does not involve the central nervous system was also proven by showing that the action of the extract was not affected by cord section plus vagotomy. Repetition of the experiments of Molitor and Pick in which the effects of subcutaneous and intralumbar injections of pituitary extract upon water diuresis were compared did not confirm them. Janssen found more marked antidiuresis to follow subcutaneous injection.

Having eliminated nervous effects, distributed by way of the spinal nerves and the vagus, from involvement in the antidiuretic effect Janssen sought to eliminate the possibility of an extrarenal, hormone mechanism. He used the technique of Miura, mentioned above, which permits the direct administration of pituitary extract to one kidney only, the other being subjected to its action later through the general circulation. In these experiments it looks as though Janssen may have made the same error which Miura had made, mistaking the initial anuria for the antidiuretic action. Since this seems to be due to a renal effect and appears immediately it is impossible to tell when the real antidiuretic affect commences. It is not certain that Janssen was dealing with the real antidiuretic action because the rate of urine secretion before the pituitary extract was given was not high and he might have observed a diuresis had the experiment been prolonged. Experiment No. 62 of Fromherz (1923) makes this criticism seem likely. Janssen seems to have thought that merely because the animal was not anesthetized pituitary extract would cause an antidiuretic action only. As has been mentioned the nature of the response to pituitary extract is not determined by whether the animal is anesthetized or not. Melville (1936b) could detect no difference in the response of the two kidneys of the dog to pituitary extract when the injection was made into the left renal artery; he concluded that such experiments gave no evidence for the view that pituitary diuresis is due to a renal action.

Theobald (1934) failed to confirm Molitor and Pick's experiments concerning the effects of pituitary extract on diuresis during chloralose narcosis. He observed as much antidiuretic effect from 0.005 unit of the extract when given to a chloralosed dog as when given to the same dog unanesthetized. He attempted to repeat Molitor and Pick's experiments on the effect of the extract when injected into the lumbar canal but it is difficult to evaluate his results since he obtained an anuria when physiological saline was injected.

There is no evidence that pituitary extract under normal conditions increases the hydration of mammalian tissues; this could occur only at the expense of the blood, and concentration of the blood has not been demonstrated. When pituitary extract is given before a "Wasserstoss" the tissues undoubtedly take up more water than when the extract is not given. It is to be expected that with the arrest of urine secretion the absorbed water will be divided between the tissues and the blood, Some experiments of Glass (1928) and of Heller and Smirk (1932) show that the muscle of rabbits contains more water following a Wasserstoss when pituitary extract is given than does corresponding control muscle, but this does not prove that the extract increased the water binding power of the muscle.

It appears probable that the hydration of frogs kept in water and receiving an injection of pituitary extract is an action peculiar to amphibia and does not indicate that the hydration of mammalian tissues is also increased. This action has been described by several investigators—Biasotti (1923), Heller, (1930a), Steggerda (1931) and Oldham (1936). Heller (1930b) and Oldham (1936) have shown that the oxytocic hormone of the extract is more potent in causing this effect than is the pressor hormone. This action is not due to the suppression of urine secretion, for Steggerda (1931) has shown that if the cloacas are ligated in a group of frogs and half the group is then injected with pitressin those receiving the injection increase in weight more than the controls. According to Boyd and Brown (1938) the hydration is greatest if the animals are kept in the dark and when the temperature is about 15°C. Boyd and Whyte (1938) have found that the loss of water in a dry environment and the elimination of injected water is slowed by pituitary extract and that both hormones are equally effective in this respect. In fish, pituitary extract does not cause the uptake of water; reptiles behave like amphibia but are less affected (Boyd and Dingwall, 1939). Steggerda (1930) has also shown that frog muscle placed in Ringer solution containing pitressin does not increase in weight any more than similar muscle placed in pure Ringer solution. In the axolotl the pressor hormone increases the absorption of sodium chloride from very dilute solution; the oxytocic hormone, on the other hand, causes loss of sodium chloride (Jorgensen *et al.* 1946).

h. The Mineral Constituents of the Urine and Blood during Antidiuresis. Do changes which occur in the composition of the urine and blood after the injection of pituitary extract throw any light upon the mechanism of the antidiuretic action? Velden (1913), in his original paper, reported that the chloride and phosphorus percentage of the urine rose during the antidiuresis, but his data show that the actual quantities excreted were not much affected. Frey and Kumpiess (1914) observed essentially the same phenomena; the absolute excretion of chloride was not increased though that of phosphorus may have been to a slight extent. The latter investigators concluded that the extract favors the excretion of salt, apparently because its concentration in the urine was increased. The only conclusion really warranted seems to be that antidiuresis occurred without affecting the amount of chloride eliminated. Konschegg and Schuster's (1915) data for a human subject show an increase in the concentration of urinary chloride but a definite decrease in the amount of chloride excreted during the antidiuretic period. C. and M. Oehme's (1918) data also show no increased chloride excretion in the period of antidiuresis in a human subject.

In spite of the foregoing evidence that pituitary extract does not increase the excretion of chlorides Fromherz (1923) showed that this is one of the most characteristic features of the action of the extract. His data leave no doubt whatever on this point, which has been confirmed by many subsequent workers. Fromherz was of the opinion that his results indicated pituitary antidiuresis to be of renal origin. According to him the extract causes diminished reabsorption of chlorides by the tubules. The fall of blood chlorides he observed fits in with such a view. This, however, does not explain the diminished excretion of water: Fromherz believed vasomotor phenomena to be involved in this feature of pituitary action.

Miura (1925) states, without giving any data, that in normal rabbits pituitary extract causes no definite changes in the plasma chloride. Buschke (1928) has observed the chloride percentage of the whole blood of normal and nephrectomized rabbits to increase and hemoglobin percentage to decrease after pituitary extract. Unfortunately one cannot tell whether the plasma chloride increased or diminished.

As stated in the section on the diuretic action of pituitary extract Stehle (1927) found that the chlorides excreted following an injection of pituitary extract consist, to a surprising extent, of potassium chloride. Because of this fact he suggested that the loss of potassium chloride from the tissues might, by a disturbance of the ionic equilibrium in the tissues, increase their water-binding capacity. However, as stated above, there is no convincing evidence that pituitary extract does increase the water-binding capacity of any mammalian tissue.

IV. Effect upon the Secretion of Urine in Nonmammalian Species

In frogs the data concerning the effect of pituitary extract upon the secretion of urine are conflicting. Brunn (1920) at first reported that when perfused through the aorta the extract caused a greater decrease in urine secretion than when perfused through the renal portal system. He thought, therefore, that the antidiuretic action was through an effect upon the glomeruli. Later (1921) he reported that in intact frogs the extract (dose not given) caused an increase in urine secretion. Burgess *et al.* (1933) could observe no antidiuretic effect in frogs with 2 units of pitressin or pituitrin per kilogram. Adolph (1936) found that 2 to 9 units of pituitary extract per kilogram slowed the urine secretion and that 20 to 100 units per kilogram caused oliguria for 2 hours, which he thought was due to the constriction of the efferent glomerular vessels.

In the fowl, according to Gibbs (1928), pituitrin caused antidiuresis and diminished blood flow through the kidney. Burgrese *et al.* (1933) observed antidiuresis following pitressin and concluded that it was due to increased tubular reabsorption.

In the alligator Burgess *et al.* (1933) observed a very intense antidiuretic action. In this species their data indicated that glomerular filtration was reduced and tubular reabsorption of water was not affected. In the catfish no antidiuretic action could be detected.

V. Effect of the Oxytocic Hormone on Urine Secretion

It was stated in the introduction to this section that there was evidence for believing that the oxytocic hormone exerts an action on urine secretion. An observation was cited above. Fraser (1937, 1942) found that the oxytocic hormone increased the urine output in hydrated and nonhydrated rats and increased the rate of water secretion in hydrated rats. The phosphorus excretion was markedly decreased. The action is stronger in rats than in dogs. The minute quantity of hormone which produces these effects warrants the belief that it is important in regulating the water balance of the organism. To what extent, if any, the action is involved in the diuretic effect of ordinary pituitary extract or even of purified preparations of the pressor hormone, which are always contaminated by the oxytocic hormone, is problematical. It will be noted that the diuretic action of the pressor hormone is accompanied by an increase in phosphate secretion, that of the oxytocic hormone by a decrease. Kuschinsky and Bundschuh (1939) have reported results similar to those of Fraser as far as water and chloride excretions are concerned. Schaumann and Schmidt (1948) have recently shown that the excretion of sodium is increased more than is that of chlorine. Boyd *et al.* (1939), using ordinary pituitary extract, observed that when the dose was very small the rate of urine secretion was augmented in rats, guinea pigs and cockerels. Some experiments of Chen and Geiling (1943), conducted for another purpose, appear to show this action.

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Errata

Page 15, heading *1* should read "Phenyl Benzo-γ-Pyrone Compounds (Flavonoids)."
Page 22, second line from the bottom, for "pitch" read "pith."
Page 28, eighth line from the bottom, for "long" read "longer."
Page 126, seventh line of the third paragraph, for "latter" read "later."
Page 166, fifth line of the fourth paragraph, for "tenmen" read "tegmen."
Page 210, seventh line of the second paragraph, for "esculetol" read "esculetin."

VITAMINS AND HORMONES, VOL VII